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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b)(2).

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**INVENTOR(s) / APPLICANT(s)**

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**TITLE OF THE INVENTION (280 characters max)**

**ANGIOPEPTIDES: NOVEL PEPTIDES THAT INDUCE ANGIOGENESIS**

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**ENCLOSED APPLICATION PARTS** *(check all that apply)*

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| ☑ Specification | *Number of Pages* | 27 | ☑ Applicant is entitled to Small Entity Status | |
| ☑ Drawing(s) | *Number of Sheets* | 33 | ☑ Other (specify) | 4 CLAIMS |

**METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT** *(check one)*

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| ☐ A check or money order is enclosed to cover the filing fees | | FILING FEE AMOUNT ($) | $ 80.- |
| ☑ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: | 50-1407 | | |

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No

☐ Yes, the name of the US Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____ Date October 27, 2003

REGISTRATION NO. (*if appropriate*) 25,457

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☐ Additional inventors are being named on separately numbered sheets attached hereto

## USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

**Confidential:** **Patent application**

# Angiopeptides: Novel peptides that induce angiogenesis

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**Scientific background**

Angiogenesis is the process of generating new capillary blood vessels involving interplay between cells and soluble factors (1).

Depending on the ultimate fate with respect to the type of vessel and vascular bed, activated endothelial cells migrate and proliferate to form new vessels, surrounded by layers of periendothelial cells, pericytes for small vessels and smooth muscle cells for large vessels.

Novel therapeutic attempts to create networks of new blood vessels to provide collateral circulation and sufficient oxygen flow follow the physiological process of new vessel formation, angiogenesis, triggered by angiogenic molecules such as Vascular Endothelium Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF), or acidic FGF/FGF-1, hypoxia inducible factor 1-alfa (HIF 1a), etc (2).

An hypoxic environment has important implications for physiological and pathological processes (3). Systemic local and intracellular homeostatic responses elicited by hypoxia include erythropoiesis by individuals who are anemic or at high altitude, neo-vascularization in ischemic myocardium, and glycolisis in cells cultured at reduced $O_2$ tension. These adaptive responses either increase $O_2$ delivery or activate alternative metabolic pathways that do not require $O_2$ (4).

In hypoxic conditions VEGF gene expression can be induced in endothelial cells and in pericytes to produce secretory forms of VEGF. Induction of VEGF gene expression occurs after hypoxia. VEGF in turn, may act on VEGF receptor-2 (Kdr) VEGF receptor-1 (Flt-1) on endothelial cells in autocrine and paracrine systems, thereby causing proliferation of endothelial cells, which may lead to angiogenesis. Basal amounts of vascular VEGF synthesized in normoxic states may promote the maintenance of microvascular homeostasis (5). Expression of mRNA VEGF receptor 1 (Flt-1) was up-regulated in peri-ischemic endothelial cells and in the infracted core of endothelial cells and periphery, with peak expression of VEGFR-1 in endothelial cells.

Gene expression of VEGF-1 is directly inducible by hypoxia, as in the case for VEGF. Twenty-four hours after hypoxia-induced VEGF gene expression and in close to the expression of the VEGFR-1 and 2 (Kdr) genes, endothelial cells started to proliferate (6,7).

Hypoxia inducible gene products that participate in these responses include erytropoietin, vascular endothelial growth factor (VEGF) and glycolitic enzymes (8).

Hypoxia can directly enhance the expression of bFGF mRNA in pericytes. Increased expression of bFGF may play an important role in the process of pericyte proliferation and differentiation of pericytes and smooth muscle cells (9).

Hypoxia inducible factor (HIF) is a master transcription factor ,which regulates oxygen homeostasis by inducing glycolisis, erytropoiesis and angiogenesis. It has been suggested that pp60c-Src mediates the hypoxic induction of VEGF expression and that the expression of v-Src enhances the expression of HIF 1 alpha which in turn up-regulates HIF 1 target genes. The presence of activated Src in many malignant tumors (e.g. colon, breast and lung cancers) has been described, and many of these tumors also express high levels of HIF 1 alfa (10). HIF also transactivates nitric oxide synthase (NOS) thereby stimulating nitric oxide. Both VEGF and NOS are induced by HIF1 in cancer cells under hypoxic conditions (11-13).

Therapeutic angiogenesis defines the intervention used to treat local hypovascularity by stimulating or inducing neovascularization for the treatment of ischemic vascular disease.

Animal studies have proven the feasibility of enhancing collateral perfusion and function via angiogenic compounds. Those experiments proved that exogenous administration of angiogenic growth factors or their genetic constructs could promote collateral vessel growth in experimental models of chronic ischemia.

Although such studies demonstrated proof of concept, additional studies raise issues that still have not been resolved; for example, the duration of exposure of the vessels to angiogenetic factors and the brief half-lives of proteins (14).

The study of synthetic peptides encompassing portions of proteins has turned into a supportive tool for understanding the molecular mechanism associated with protein biological functions.

The use of short peptides, constructed from specific regions of human FGF and VEGF that have the potential to efficiently agonize or antagonize the biological functions of the growth factors family members have been described (15).

The new technology, combinatorial library, is employed to study almost any biological target and can be used to increase fundamental understanding of cellular function and signaling pathway. It can also be used for discovery as well as optimization of drug leads.

Several groups have reported the use of intact cells to screen phage display peptide library to identify cell surface binding peptides (16).

It was reported that a peptide library was screened on human endothelial cells stimulated with VEGF to construct a peptide-based ligand receptor map of the VEGF family (17).

Another study has described the screening of a 12-mer phage display peptide library on VEGF-2 receptor protein (18).

Our approach focused on screening a phage display peptide library on endothelial cells under physiological conditions in order to reveal novel cell surface binding peptides that will trigger angiogenic processes that include vascularization, re-vascularization and neo-vascularization.

**Material and Methods**

**A. Human Umbilical Vein Endothelial Cells (HUVEC):**

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion as previously described (Jaffe et al. 1973). HUVEC were cultured with M199 supplemented with 20% FCS, 10000 units penicillin, 10mg/ml streptomycin sulfate, 10mg/ml neomycin sulfate (Biological Industries, kibbutz Beit Haemek, Israel), 25ug/ml endothelial cell growth supplement (Biomedical Technologies, MA, USA) and 5U/ml Heparin (SIGMA, Rehobot, Israel). HUVEC were harvested with Trypsin (0.25%) EDTA (0.05%) (Biological Industries, kibbutz Beit Haemek, Israel) and incubated on 60mm petri-dishes coated with 1%gelatin for 24. After then, cells were washed and incubated with M199 supplemented with 10% FCS. HUVEC or in short, EC, were subjected to three different treatment groups: a) EC without treatment, b) EC subjected to 3 hours hypoxia, c) EC subjected to 24 hours hypoxia.

Monolayers of EC after treatment were washed with PBS and dried over night. Cells were rehydrated with PBS containing 5% FCS and 0.1% sodium azide and kept at 4°C until the process of biopanning .

**B. Hypoxia conditions:**

EC cells were subjected to hypoxia for 3 or 24 hours with a mix gas of 94% Nitrogen + 5% $CO_2$ + 1% $O_2$ in a hypoxia chamber (Billups-Rothenberg, CA, USA).

**C. Phage Display Peptide Library:**

The Random Phage Display Peptide Library employed in this study was purchased from New England Biolabs, Inc. (MA, USA). The phage display library is based on a combinatorial library of random peptide 12-mers fused to a minor coat protein (pIII) of M13 phage. The displayed peptide12-mers are expressed at the N-terminus of pIII. The library consists of about $2.7x10^9$ electroporated sequences amplified once to yield 20 copies of each sequence in 10ul of the supplied phage.
The Phage display peptide library was subjected to 5 rounds of affinity positive selection (biopanning) in screening against three different treated HUVEC endothelial cells (EC): a) EC without treatment, b) EC after 3 hours hypoxia, c) EC after 24 hours hypoxia . Each positive selection was preceded by a negative selection on human PBL. Each round of biopanning was characterized by elution of the bound phages with 0.2% M glycine- HCl. The unbound phages were incubated on a second EC plate; this procedure was executed 3 times. Phages of the 3 eluted phages were unificated for the second round of biopanning and so on. After the fifth round of biopanning 40 individual clones from each group of cells screened were isolated, all together 120 individual clones were obtained.

**D. Screening of positive clones by ELISA:**

Screening of positive clones from each group was done by an ELISA method. For this purpose 96 well plates with 20000/well of EC, EC after 3h hypoxia or 24 hs hypoxia were prepared. In addition plates with human PBL were prepared as controls. Plate re-hydration was done with PBS+3% BSA over night at 4°C. Then,

wells were washed 3 times with PBS. Phages from each of the 120 clones isolated were dispered on the ELISA plates in 3 different concentrations: $10^{10}$, $10^{9}$ and $10^{8}$ phages per well were incubated for 2 hs at room temperature . Then, plates were washes 3 times with PBS+0.05% tween and 3 times with DDW. Anti-M13 HRP antibody (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK) in a dilution of 1:5000 was added for 2 hs. After washes 100µl tetramethyl benzidine liquid substrate ( DAKO TMB substrate chromogen, DAKO corporation, CA, USA.) was added for 15 minutes and the reaction was stopped with 1M HCl. Plates were read at 450 nm in an ELISA reader.

**E. Proliferation of EC with Phage Display Peptides**

EC (40000cells/well) were seeded on 24 well gelatin (1%) - coated plates cultured with M199 medium supplemented with 20% FCS, 10000 units penicillin, 10mg/ml streptomycin sulfate, 10mg/ml neomycin sulfate (Biological Industries, kibbutz Beit Haemek, Israel), 25ug/ml endothelial cell growth supplement (Biomedical Technologies, MA, USA) and 5U/ml Heparin (SIGMA, Rehobot, Israel). After 24 hs, cells were washed and incubated with serum free media for another 24 hs. Then, $10^{6}$ phages were added per each well and plates were incubated for 24 hs. Abortive Phages ,without an exposed peptide, were used as negative controls and are designated NO. 2µCi/well of thymidine (SIGMA, Rehovot, Israel) were added for the last 6 hs of incubation and then cells were fixed with TCA 10% over night at 4°C. Cells were washed with absolute ethanol and then incubated for 15 min at 37°C with 300µl/well of 0.5M NaOH to lyse cells. Lysates were transferred to a scintillation vial with 2 ml scintillation liquid Ultima Gold (Packard Bioscience, Meriden, USA) and counted in a ß counter. Results were obtained as cpm/ 1min.

**F. Endothelial Cell Migration by selected peptide phages:**

Endothelial cell migration was evaluated by the Chemicon QCM 96-well Migration Assay (Chemicon International, CA, USA) according to manufacturer instructions.
This kit utilizes a membrane with an 8µm pore size. Migratory cells on the bottom of the insert membrane are dissociated from the membrane when incubated with cell

detachment buffer provided by the kit. These cells are subsequently lysed and detected by a molecular probe CyQuant GR dye. This green fluorescent dye exhibits fluorescent enhancement when bound to cellular nucleic acid. For the migration assay, endothelial cells from passage 3 were incubated on gelatin coated plates in M199 medium free serum for 24 hs. After trypsinization, 20000 EC were incubated in each of the 96 wells migration chamber. $10^5$ or $10^6$ phages were added to the feeder tray for chemoattractant assay. For activation migration assays, $10^5$ or $10^6$ phages were incubated with the EC cells in the migration chamber. Phages without an exposed peptide were used as negative controls and are designated NO. Time of incubation for both assays was 5 hs.
Results were determined by a Fluorescent ELISA reader at 480/520nm.

## G. Aortic rings with Phage Display Peptides

Human mammary or radial artery was removed of adventitia and cut into 1mm long rings. The bottom of each well of sterile 96-well plate was coated with 20 µg of fibronectin (Biological Industries, Kibbutz Beit Ha Hemek, Israel) and the rings were positioned in the center of each well containing 150 ml of Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Kibbutz Beit Ha Hemek, Israel) containing 10% FCS. $10^6$ selected cloned phages were added to each well. Phages without an exposed peptide (NO)were used as negative controls. Plates were incubated at 37°C with 5% $CO_2$ for 7 days. Arterial rings were removed and the extent of cell proliferation was estimated by an XTT assay (Biological Industries, Kibbutz Beit Ha Hemek, Israel) according to manufacturer instructions.

## H. Identification of DNA sequences from selected phages

DNA from all isolated selected clones was purified by incubation with iodide buffer and ethanol (according to manual instructions, NEB, MA, USA). This rapid procedure produces template of sufficient purity for automated sequencing with dye labeled dideoxynucleotides. The –96 gIII (NEB, MA, USA) sequencing primer was utilized for automated sequencing by the Sequencing Unit of Tel Aviv University.

## I. Peptides Synthesis

Peptides were synthesized by SynPep (Dublin, CA, USA). HPLC purity from each peptide synthezised was > than 97%. All the peptides were dissolved in water in exception of QF that was dissolved in 50% water + 50% acetonitrile.

## J. Fluorescein labeling of synthetic peptides

Fluorescein Isothiocyanate (FITC) (Pierce, Rockford, IL) is an amino reactive probe that reacts under alkaline condition with primary amines to form a stable fluorescent derivative.

Synthetic peptides in a concentration of 1mg/ml, were diluted with 0.5M bicarbonate buffer (pH 9.5). 12.5µl of FITC in a concentration of 10mg/ml was added per mg of peptide and incubated on a test tube rocker in the dark for 2 hs. Then, 0.1ml of 1.5M hydroxilamine was added per ml of reaction and incubated on a test tube rocker for 30 minutes at room temperature. Unbound FITC was removed by dialysis.

## K. FACS analysis of peptide binding to Endothelial Cells

EC cultured with M199+10%FCS or EC exposed to 24 hs hypoxia, were harvested by trypsin and samples of 100,000 cells were suspended in PBS+5%FCS+0.1% Na azide. Cells were stained with 1-6µg labeled peptides for 2 hs on ice in dark. Then, cells were washed twice with PBS. Samples were analyzed by Fluorescence Activated Cell Sorter (FACScan Beckton Dickinson, CA, USA).

## L. Proliferation of EC

EC (40000cells/well) were seeded on 24 well plates coated with 1% gelatin in M199 medium supplemented with 10% FCS. After 22 h of incubation with different concentrations of synthetic peptides (0.01, 0.1,0.5, 1 and 10ng/ml) ,[$H^3$]thymidine 2µCi/well (SIGMA, Rehovot, Israel) was added for 6 hs. Plates were washed 5 times

with PBS and than incubated for 15 min at 37°C with 300µl/well of 0.5M NaOH. Lysated cells were transferred to a scintillation vial with 2 ml scintillation liquid Ultima Gold (Packard Bioscience, Meriden, USA) and counted in a ß counter. Results were obtained as cpm/ 1min.

## M. Endothelial Cell Migration

Endothelial cell migration was evaluated by the Chemicon QCM 96-well Migration Assay (Chemicon International, CA, USA) according to manufacturer instructions. This kit utilizes an 8µm pore size membrane. Migratory cells on the bottom of the insert membrane are dissociated from the membrane when incubated with cell detachment buffer provided by the kit. These cells are subsequently lysed and detected by a molecular probe CyQuant GR dye. This green fluorescent dye exhibits fluorescent enhancement when bound to cellular nucleic acid. For the migration assay, endothelial cells from passage 3 were incubated on gelatin in M199 free serum for 24 hs. After trypsinization, 25000 EC were incubated in the migration chamber. Synthetic peptides were added at 5, 10, 20 and 50 ng/ml in the feeder tray for chemoattractant migration assay. For activation migration assays, synthetic peptides at 0.1, 1 a 10ng/ml were incubated with the cells in migration chamber. Time of incubation in both cases was 5 hs.
Results were determined in a Fluorescent ELISA reader at 480/520nm.

## N. Sprouting of Aortic Rings by Peptides

Human mammary or radial artery was removed of adventitia and cut into1mm long rings. The bottom of each well of sterile 96-well plate was coated with 20 µg of fibronectin (Biological Industries, Kibbutz Beit Ha Hemek, Israel) and the rings were positioned in the center of each well containing 150 ul of Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Kibbutz Beit Ha Hemek, Israel) suplemented with 10% FCS. Peptides at 1, 10,100 and 1000 ng/ml were added to each well with aortic ring. Plates were incubated at 37°C with 5% $CO_2$ for 7 days. Arterial rings were

removed and cell proliferation was estimated by the XTT assay (Biological Industries, Kibbutz Beit Ha Hemek, Israel) according to manufacturer instructions.

## O. A mouse-ear model- In vivo angiogenesis

Ear angiogenesis studies were a modification of an approach described previously (Pettersson, 2000). Synthetic peptides in a concentration of 1, 10 and 20 µg/15 µl per mouse were injected sub-cutaneously into the ears of nude mice and Balb/C mice. Contralateral ears were injected only with PBS. Digital photographs were obtained after 4, 6 and 20 days after injection. Histological examination of the ears was performed and slides were stained with hematoxolin-eosin.

## P. Rat Ischemic hind-limb model and Laser-Doppler Imager analysis

We used a rat ischemic hind limb model for evaluation of the in vivo potential of angiogenesis induced by the selected synthetic peptides. Ischemia was created in the rat left hind limb by ligation the femoral artery. The right hind limb served as a control.

A day after the operation each of the peptides was injected into two sites close to the ligation and one site distal to the ligation. Each rat was treated with each of the peptides in a total amount of 600ug.

The blood flow was measured using a Laser Doppler Blood Flow analyzer (moorLDI, Moor Instrument, Wilmington, Delaware) at 4 time points (at days 2, 6, 9 and 13). The average perfusion of each limb was computed and blood flow was expressed as the ischemic (left) / control (right) blood flow ratio.

## Results

Vascularization and re-vascularization are complex biological processes themselves comprised of multiple sequential and simultaneous interacting stages. There are several steps in this process where the test peptides should exert their influence. The peptides must be capable of specific binding to certain target cells, under the correct conditions; they must signal proliferation of cells necessary for vascularization; they must induce migration of these cells, and; the peptides must result in tube formation and other processes involved in creating vascular structures. Further, the additional influence of factors such as peptide concentration and environmental stresses, such as hypoxia, must be considered.

We do not expect that each peptide will be necessarily able to modulate every step. Quite the contrary; in such a complex process, we expect certain of the peptides to each be optimized for one or more steps. The peptides almost certainly act in concert to control vascularizaton. Our role is to determine how they do this.

### Statistical and graphical methods

Statistical analysis generally consisted of analysis of variance (ANOVA), with appropriate post-hoc tests, generally Dunnett's for comparison to a control or Tukey-Kramer HSD for multiple comparisons. Results are considered statistically significant at $P<0.05$.

Several experiments could not be analyzed statistically, due to the difficulty of producing, at this time, the requisite number of replicates necessary for formal analyses. These studies are presented in graphical format only.

Figures are often presented after adjustment for a blank or other negative control, for clarity and to enhance visual comparisons between treatment effects. However, some statistical tests may have been performed against these controls, and are so noted.

**Phage – bound Peptides**

We subjected a phage display peptide library to 5 rounds of affinity positive selections (biopanning) in screening against endothelial cells (EC) under physiological conditions and hypoxia. We used negative selection on lymphocytes. After the fifth round of biopanning, 40 individual clones from each EC were selected, isolated and the purified DNA was sequenced.

1. The second step of selection of peptide bound phages was done by ELISA method using EC and lymphocytes coated plates as controls. Phages attached to selected peptides were detected by anti-M13 HRP antibody followed by a chromogen substrate and determined by ELISA reader. As can be seen in **Fig. 1,** fifteen different peptide bound phages at $10^9$ and $10^{10}$ per well were screened on 4 different EC preparations; EC at normal conditions and EC after 3,6,and 24h of hypoxia.

   ANOVA on $10^{10}$ selected phages on EC treated cells indicated statistically significant differences between binding of NO phages and binding of all peptide-bound phages, as indicated in the figure below.
   ANOVA on $10^9$ selected phages on EC treated cells indicated statistically significant differences between binding of NO phages and binding of certain peptides-bound phages, as indicated in the table and figure below.

Peptide-bound phages which bound significantly:

| Peptide | cells |
|---|---|
| SP | EC |
| SP | H3 |
| TR | EC |
| TR | H3 |
| TR | H6 |
| TR | H24 |
| VL | EC |
| VL | H3 |
| VL | H6 |
| VL | H24 |
| YR | EC |
| YR | H3 |
| YR | H6 |
| YR | H24 |

2. **Fig. 2** demonstrates the number of identical DNA sequences obtained from the positive selected phages of about 120 sequences obtained from screening three groups of ECs.

3. Since we limited the number of peptides that will further be synthesized to 6, we chose 6 of the DNA sequences to obtain their peptides. **Fig.3** shows the DNA and protein sequences of the 6 chosen peptides.

Induction of angiogenesis is a complex of biological processes comprised of multiple sequential and simultaneous interacting stages. There are several steps in this process where the test peptides should exert their influence invitro such as binding to EC , proliferation of EC , migration of EC. Invivo, the peptides should induce vascularization, re-vascularization and neo-vascularization in normal and ischemic organs in animal models.

4. We first tested the angiogenic activity of the phage bound selected peptides: EC were incubated with $10^6$ phages. **Fig. 4** demonstrates increased proliferation induced by the phages presenting peptides over proliferation with the empty control phages.

5. Phage display selected peptides were tested to induce migration of endothelial cell in migration chambers as described in M&M. In **Fig. 5** phages bound peptides were incubated at two different concentrations with EC. As can be seen QF and LT induced migration of the activated EC. Placing the phage display peptides at two different concentrations on the feeder tray revealed the ability of QF and LT (of the 6 selected phage display peptides) to induce migration as chemoattractants at $10^5$ phages per well. $10^6$ phages bound to QF, SP TR and LT induced migration as chemoattractants (**Fig.6**)

6. Aortic rings were tested for the ability to sprout in the presence of phage bound selected peptides. **Fig. 7.** demonstrates proliferation of cells originating from the aortic rings.
   ANOVA comparing the different phage display peptides indicates an overall clear difference between them (P=0.0003), indicating differences in proliferation of cells derived from the aortic rings, as illustrated in the figure below. Post-hoc tests indicate statistically significant differences between peptide VL and the S24 empty phage control.

**Synthetic Peptides**

We have obtained six synthetic peptides each composed of 12 amino acids for further studies. The peptides **LT, QF, SP, TR, VL, and YR.**

7. We first tested the specificity of the synthetic 12mer peptides by binding to EC and lymphocytes. We labeled the peptides by FITC and analysed the binding by FACS analysis. The results (**Fig. 8**) obtained indicate that the peptides bind specifically to endothelial cells, and *not* to lymphocytes. Increasing concentrations of peptides resulted in increased binding to EC (94-96% binding) and increased intensity of binding of the fluorescent-labeled peptides to EC (**Fig 9**).

8. Peptides binding to EC and to EC after hypoxia conditions is seen in **Fig. 10** and **11.** As can be seen, LT and SP showed increased binding to EC exposed to hypoxia while other peptides had similar intensity of binding to EC and EC after hypoxia

9. We tested the effect of different concentrations of the peptides on proliferation of EC **(Fig. 12a)**. EC were seeded on 24 well plates coated with gelatin in M199 supplemented with 10% FCS. After 24 hrs synthetic peptides were added in various concentrations. 2μCi/well of $H^3$thymidine was added for 6 hrs followed by 5 washes with PBS. Cells were harvested and uptake of $H^3$thymidine was determined by scintillation ß counter.

Different concentrations of the different peptides have varying effects on proliferation, as can be seen in **fig. 12a.** Post-hoc tests, summarized in the table, comparing peptide-induced proliferation against a blank control illustrate this. Note that YR is the most effective in inducing proliferation, and that the 1 ng/ml dosage the most effective for almost all the peptides.

Proliferation of peptides at different concentrations which are significantly different from blank controls. Statistical significance indicated by "+"

| Peptide | Concentration (ng/ml) | | | |
|---|---|---|---|---|
| | 0.1 | 0.5 | 1 | 5 |
| LT | | | + | |
| QF | + | | + | |
| SP | | | + | |
| TR | | | + | |
| VL | + | | | |
| YR | + | + | + | |

A comparison of peptides incubated for 24 hours with EC indicates that all of the peptides were statistically significantly able to induce increases in proliferation compared to controls. When the peptides were similarly incubated for only 6 hours, only YR was able to induce a significant increase in proliferation relative to control. **Fig 12.b**

Some of the peptides, under the proper concentrations, can strongly induce proliferation under conditions of extended (18 hour) EC hypoxia. **Fig.12c**

illustrates proliferation induced by 10 ng/ml peptide concentrations. Hypoxia produced statistically significantly greater proliferation in combination with these peptides than the peptides with normally oxygenated cells, as indicated by the asterisks (*) in the figure.

10. Migration of endothelial cells can be considered as a function, and thus an indication, of activation and attraction by peptides. We tested the effect of the peptides on endothelial cell migration using the Chemicon QCM 96-well Migration kit (Chemicon International, CA, USA). This kit utilizes an 8μm pore size membrane. Migratory cells on the bottom of the insert membrane are dissociated from the membrane by detachment buffer, lysed and detected by a fluorescent dye bound to nucleic acid. Synthetic peptides were added at 5, 10, 20 and 50 ng/ml to endothelial cells incubated in the feeder tray for 5 h to induce migration by chemical attraction. This can be observed in **Fig.13a** for LT and SP, where there is a clear dose-dependency for induced migration. The effect appears to reach a plateau at high concentrations, which would be predicted on the basis of pharmacokinetics. The exact phamacologic profile of this attraction requires further study. A smaller effect was noted using VL and TR as chemoattractants. **Fig.13b**

Experiment performed with the test peptides demonstrates their effectiveness in activating migration. **Fig. 14** illustrates that each of the test peptides, within a 5 hour span, induces statistically significantly more endothelial cell migration than control endothelial cells without the peptide present. By 15 hours, however, the migration of cells has been reduced, so that no statistical difference is seen between any peptide-treated cells and control epithelial cells.

11. Aortic Rings proliferation by peptides: As described before for the phage bound peptides, we repeated the experiments on aortic rings sprouting and cell proliferation by addition to the cultured aortic rings purified synthetic peptides at different concentrations . Four peptides (QF, YR, LT, and VL) were compared for their ability to induce cell proliferation in aortic rings. After

correction for control (blank) optical density, **Fig.15** illustrates clear differences between the peptides, with decided effects of concentration.

12. In vivo angiogenesis : Ear angiogenesis model was used to evaluate the in vivo angiogenic effect of the selected peptides. In 3 experiments, one and ten micrograms of synthetic peptides were injected sub-cutaneously into the ears of Balb/C mice. Control ears were injected only with PBS. Digital photographs were obtained at different times, post injection. Increased number of blood vessels was noted in the ears of mice injected with 10ug of LT , YR, QF and SP (**Fig. 16**). **Histological examination** of stained sections of the ears revealed an increase in the number of blood vessels and the appearance of neo-vascularizations in peptide injected ears (**Fig. 17** and **PPt file : Histology of mice ears injected with peptides)**

13. Rat ischemic hind-limb model and laser-Doppler analysis
We used the ischemic hind limb model. Ischemia was created in the rat left hind limb by ligating the femoral artery. The right hind limb served as a control. A day after the operation 600ug of peptide was injected into two sites close to the ligation and one site distal to the ligation. The blood flow was measured using a Laser Doppler Blood Flow Imager (MoorLDI, Moor Instrument, Wilmington, Delaware) at 4 time points (at days 2, 6, 9 and 13). The laser beam reflected from moving red blood cells in capillaries as in arteriols and venoules is detected and processed to provide a flux value. The information is color coded to provide a map of tissue perfusion (**Fig. 18**). The average perfusion of each limb was computed and blood flow was expressed as the ischemic (left) / control (right) blood flow ratio. Results of 2 repeated experiments in which SP and TR were injected to the ischemic leg show a constant higher blood flow ratio compared to that of PBS injected rats. **Fig.19.**

**Commercial Potential**

Cardiovascular disease is the leading cause of mortality in the United States, Europe, and Israel. In the United States approximately one million deaths per year are attributed to the cardiac causes of which fifty percent are attributed to Coronary Artery Disease (CAD). The major morbidity from CAD is a result of obstructive coronary artery narrowing and the resultant myocardial ischemia. CAD affects more than 13 million people, and its annual economic burden is in excess of US$60 billion US.

Mechanical revascularization of obstructive coronary stenoses by percutaneous techniques including percutaneous transluminal angioplasty and stent implantation is used to restore normal coronary artery blood flow. In addition, Coronary artery occlusion bypass surgery is performed using arterial and venous conduits as grafts into the coronary arterial tree. These treatment modalities have significant limitations in individuals with diffuse atherosclerotic disease, severe small vessel coronary artery disease, diabetic patients, or individuals having already undergone surgical or percutaneous procedures. Therapeutic angiogenesis defines the intervention to treat local hypovascularity by stimulating or inducing neovascularization for the treatment of ischemic vascular disease.

The concept of therapeutic angiogenesis is based on the premise that the existing potential for vascular growth inherent in vascular tissue can be utilized to promote the development of new blood vessels under the influence of the appropriate growth factors (19).

Novel therapeutic attempts to create networks of new blood vessels to provide collateral circulation and sufficient oxygen flow follow the physiological process of new vessel formation. Angiogenesis is triggered by angiogenic molecules such as Vascular Endothelium Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF), or acidic FGF/FGF-1, hypoxia inducible factor 1-alfa (HIF 1a), etc (2).

Our approach on novel angiogenic molecules has the potential for rapid clinical application, with the promise of relief from a host of medical disorders.

The term "peptide" as used herein encompasses native peptides (either degradation products, synthetically synthesized peptides or recombinant peptides) and peptidomimetics (typically, synthetically synthesized peptides), as well as as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications rendering the peptides more stable while in a body or more capable of penetrating into cells. Such modifications include, but are not limited to N terminus modification, C terminus modification, peptide bond modification, including, but not limited to, CH2-NH, CH2-S, CH2-S=O, O=C-NH, CH2-O, CH2-CH2, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further details in this respect are provided hereinunder.

Peptide bonds (-CO-NH-) within the peptide may be substituted, for example, by N-methylated bonds (-N(CH3)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-), ketomethylen bonds (-CO-CH2-), α-aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH2-NH-), hydroxyethylene bonds (-CH(OH)-CH2-), thioamide bonds (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), peptide derivatives (-N(R)-CH2-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom.

These modifications can occur at any of the bonds along the peptide chain and even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

In addition to the above, the peptides of the present invention may also include one or more modified amino acids or one or more non-amino acid monomers (e.g. fatty acids, complex carbohydrates etc).

As used herein in the specification and in the claims section below the term "amino acid" or "amino acids" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including,

for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acids including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids.

Tables 1 and 2 below list naturally occurring amino acids (Table 1) and non-conventional or modified amino acids (Table 2) which can be used with the present invention.

***Table 1***

| Amino Acid | Three-Letter Abbreviation | One-letter Symbol |
|---|---|---|
| alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glutamic Acid | Glu | E |
| glycine | Gly | G |
| Histidine | His | H |
| isoleucine | Iie | I |
| leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| tryptophan | Trp | W |
| tyrosine | Tyr | Y |
| Valine | Val | V |
| Any amino acid as above | Xaa | X |

**Table 2**

| Non-conventional amino acid | Code | Non-conventional amino acid | Code |
|---|---|---|---|
| α-aminobutyric acid | Abu | L-N-methylalanine | Nmala |
| α-amino-α-methylbutyrate | Mgabu | L-N-methylarginine | Nmarg |
| aminocyclopropane-carboxylate | Cpro | L-N-methylasparagine | Nmasn |
| | | L-N-methylaspartic acid | Nmasp |
| aminoisobutyric acid | Aib | L-N-methylcysteine | Nmcys |

| | | | |
|---|---|---|---|
| aminonorbornyl-carboxylate | Norb | L-N-methylglutamine | Nmgin |
| | | L-N-methylglutamic acid | Nmglu |
| cyclohexylalanine | Chexa | L-N-methylhistidine | Nmhis |
| cyclopentylalanine | Cpen | L-N-methylisolleucine | Nmile |
| D-alanine | Dal | L-N-methylleucine | Nmleu |
| D-arginine | Darg | L-N-methyllysine | Nmlys |
| D-aspartic acid | Dasp | L-N-methylmethionine | Nmmet |
| D-cysteine | Dcys | L-N-methylnorleucine | Nmnle |
| D-glutamine | Dgln | L-N-methylnorvaline | Nmnva |
| D-glutamic acid | Dglu | L-N-methylornithine | Nmorn |
| D-histidine | Dhis | L-N-methylphenylalanine | Nmphe |
| D-isoleucine | Dile | L-N-methylproline | Nmpro |
| D-leucine | Dleu | L-N-methylserine | Nmser |
| D-lysine | Dlys | L-N-methylthreonine | Nmthr |
| D-methionine | Dmet | L-N-methyltryptophan | Nmtrp |
| D-ornithine | Dorn | L-N-methyltyrosine | Nmtyr |
| D-phenylalanine | Dphe | L-N-methylvaline | Nmval |
| D-proline | Dpro | L-N-methylethylglycine | Nmetg |
| D-serine | Dser | L-N-methyl-t-butylglycine | Nmtbug |
| D-threonine | Dthr | L-norleucine | Nle |
| D-tryptophan | Dtrp | L-norvaline | Nva |
| D-tyrosine | Dtyr | α-methyl-aminoisobutyrate | Maib |
| D-valine | Dval | α-methyl-γ-aminobutyrate | Mgabu |
| D-α-methylalanine | Dmala | α-methylcyclohexylalanine | Mchexa |
| D-α-methylarginine | Dmarg | α-methylcyclopentylalanine | Mcpen |
| D-α-methylasparagine | Dmasn | α-methyl-α-napthylalanine | Manap |
| D-α-methylaspartate | Dmasp | α- methylpenicillamine | Mpen |
| D-α-methylcysteine | Dmcys | N-(4-aminobutyl)glycine | Nglu |
| D-α-methylglutamine | Dmgln | N-(2-aminoethyl)glycine | Naeg |
| D-α-methylhistidine | Dmhis | N-(3-aminopropyl)glycine | Norn |
| D-α-methylisoleucine | Dmile | N- amino-α-methylbutyrate | Nmaabu |
| D-α-methylleucine | Dmleu | α-napthylalanine | Anap |
| D-α-methyllysine | Dmlys | N-benzylglycine | Nphe |
| D-α-methylmethionine | Dmmet | N-(2-carbamylethyl)glycine | Ngln |
| D-α-methylornithine | Dmorn | N-(carbamylmethyl)glycine | Nasn |
| D-α-methylphenylalanine | Dmphe | N-(2-carboxyethyl)glycine | Nglu |
| D-α-methylproline | Dmpro | N-(carboxymethyl)glycine | Nasp |

| | | | |
|---|---|---|---|
| D-α-methylserine | Dmser | N-cyclobutylglycine | Ncbut |
| D-α-methylthreonine | Dmthr | N-cycloheptylglycine | Nchep |
| D-α-methyltryptophan | Dmtrp | N-cyclohexylglycine | Nchex |
| D-α-methyltyrosine | Dmty | N-cyclodecylglycine | Ncdec |
| D-α-methylvaline | Dmval | N-cyclododeclglycine | Ncdod |
| D-α-methylalnine | Dnmala | N-cyclooctylglycine | Ncoct |
| D-α-methylarginine | Dnmarg | N-cyclopropylglycine | Ncpro |
| D-α-methylasparagine | Dnmasn | N-cycloundecylglycine | Ncund |
| D-α-methylasparatate | Dnmasp | N-(2,2-diphenylethyl)glycine | Nbhm |
| D-α-methylcysteine | Dnmcys | N-(3,3-diphenylpropyl)glycine | Nbhe |
| D-N-methylleucine | Dnmleu | N-(3-indolylyethyl) glycine | Nhtrp |
| D-N-methyllysine | Dnmlys | N-methyl-γ-aminobutyrate | Nmgabu |
| N-methylcyclohexylalanine | Nmchex | D-N-methylmethionine | Dnmmet |
| D-N-methylornithine | Dnmorn | N-methylcyclopentylalanine | Nmcpen |
| N-methylglycine | Nala | D-N-methylphenylalanine | Dnmphe |
| N-methylaminoisobutyrate | Nmaib | D-N-methylproline | Dnmpro |
| N-(1-methylpropyl)glycine | Nile | D-N-methylserine | Dnmser |
| N-(2-methylpropyl)glycine | Nile | D-N-methylserine | Dnmser |
| N-(2-methylpropyl)glycine | Nleu | D-N-methylthreonine | Dnmthr |
| D-N-methyltryptophan | Dnmtrp | N-(1-methylethyl)glycine | Nva |
| D-N-methyltyrosine | Dnmtyr | N-methyla-napthylalanine | Nmanap |
| D-N-methylvaline | Dnmval | N-methylpenicillamine | Nmpen |
| γ-aminobutyric acid | Gabu | N-(*p*-hydroxyphenyl)glycine | Nhtyr |
| L-*t*-butylglycine | Tbug | N-(thiomethyl)glycine | Ncys |
| L-ethylglycine | Etg | penicillamine | Pen |
| L-homophenylalanine | Hphe | L-α-methylalanine | Mala |
| L-α-methylarginine | Marg | L-α-methylasparagine | Masn |
| L-α-methylaspartate | Masp | L-α-methyl-*t*-butylglycine | Mtbug |
| L-α-methylcysteine | Mcys | L-methylethylglycine | Metg |
| L-α-methylglutamine | Mgln | L-α-methylglutamate | Mglu |
| L-α-methylhistidine | Mhis | L-α-methylhomo phenylalanine | Mhphe |
| L-α-methylisoleucine | Mile | N-(2-methylthioethyl)glycine | Nmet |
| D-N-methylglutamine | Dnmgln | N-(3-guanidinopropyl)glycine | Narg |
| D-N-methylglutamate | Dnmglu | N-(1-hydroxyethyl)glycine | Nthr |
| D-N-methylhistidine | Dnmhis | N-(hydroxyethyl)glycine | Nser |
| D-N-methylisoleucine | Dnmile | N-(imidazolylethyl)glycine | Nhis |

| | | | |
|---|---|---|---|
| D-N-methylleucine | Dnmleu | N-(3-indolylyethyl)glycine | Nhtrp |
| D-N-methyllysine | Dnmlys | N-methyl-γ-aminobutyrate | Nmgabu |
| N-methylcyclohexylalanine | Nmchex | D-N-methylmethionine | Dnmmet |
| D-N-methylornithine | Dnmorn | N-methylcyclopentylalanine | Nmcpen |
| N-methylglycine | Nala | D-N-methylphenylalanine | Dnmphe |
| N-methylaminoisobutyrate | Nmaib | D-N-methylproline | Dnmpro |
| N-(1-methylpropyl)glycine | Nile | D-N-methylserine | Dnmser |
| N-(2-methylpropyl)glycine | Nleu | D-N-methylthreonine | Dnmthr |
| D-N-methyltryptophan | Dnmtrp | N-(1-methylethyl)glycine | Nval |
| D-N-methyltyrosine | Dnmtyr | N-methyla-napthylalanine | Nmanap |
| D-N-methylvaline | Dnmval | N-methylpenicillamine | Nmpen |
| γ-aminobutyric acid | Gabu | N-(*p*-hydroxyphenyl)glycine | Nhtyr |
| L-*t*-butylglycine | Tbug | N-(thiomethyl)glycine | Ncys |
| L-ethylglycine | Etg | penicillamine | Pen |
| L-homophenylalanine | Hphe | L-α-methylalanine | Mala |
| L-α-methylarginine | Marg | L-α-methylasparagine | Masn |
| L-α-methylaspartate | Masp | L-α-methyl-*t*-butylglycine | Mtbug |
| L-α-methylcysteine | Mcys | L-methylethylglycine | Metg |
| L-α-methylglutamine | Mgln | L-α-methylglutamate | Mglu |
| L-α-methylhistidine | Mhis | L-α-methylhomophenylalanine | Mhphe |
| L-α-methylisoleucine | Mile | N-(2-methylthioethyl)glycine | Nmet |
| L-α-methylleucine | Mleu | L-α-methyllysine | Mlys |
| L-α-methylmethionine | Mmet | L-α-methylnorleucine | Mnle |
| L-α-methylnorvaline | Mnva | L-α-methylornithine | Morn |
| L-α-methylphenylalanine | Mphe | L-α-methylproline | Mpro |
| L-α-methylserine | mser | L-α-methylthreonine | Mthr |
| L-α-methylvaline | Mtrp | L-α-methyltyrosine | Mtyr |
| L-α-methylleucine | Mval | L-N-methylhomophenylalanine | Nmhphe |
| N-(N-(2,2-diphenylethyl) carbamylmethyl-glycine | Nnbhm | N-(N-(3,3-diphenylpropyl) carbamylmethyl(1)glycine | Nnbhe |
| 1-carboxy-1-(2,2-diphenyl ethylamino)cyclopropane | Nmbc | | |

***Table 2 Cont.***

Since the present peptides are preferably utilized in therapeutics or diagnostics which require the peptides to be in soluble form, the peptides of the present invention

preferably include one or more non-natural or natural polar amino acids, including but not limited to serine and threonine which are capable of increasing peptide solubility due to their hydroxyl-containing side chain.

The peptides of the present invention are preferably utilized in a linear form, although it will be appreciated that in cases where cyclicization does not severely interfere with peptide characteristics, cyclic forms of the peptide can also be utilized.

Cyclic peptides can either be synthesized in a cyclic form or configured so as to assume a cyclic form under desired conditions (e.g., physiological conditions).

For example, a peptide according to the teachings of the present invention can include at least two cysteine residues flanking the core peptide sequence. In this case, cyclization can be generated via formation of S-S bonds between the two Cys residues. Side-chain to side chain cyclization can also be generated via formation of an interaction bond of the formula -(-CH2-)n-S-CH2-C-, wherein n = 1 or 2, which is possible, for example, through incorporation of Cys or homoCys and reaction of its free SH group with, e.g., bromoacetylated Lys, Orn, Dab or Dap. Furthermore, cyclization can be obtained, for example, through amide bond formation, e.g., by incorporating Glu, Asp, Lys, Orn, di-amino butyric (Dab) acid, di-aminopropionic (Dap) acid at various positions in the chain (-CO-NH or -NH-CO bonds). Backbone to backbone cyclization can also be obtained through incorporation of modified amino acids of the formulas H-N((CH2)n-COOH)-C(R)H-COOH or H-N((CH2)n-COOH)-C(R)H-NH2, wherein n = 1-4, and further wherein R is any natural or non-natural side chain of an amino acid.

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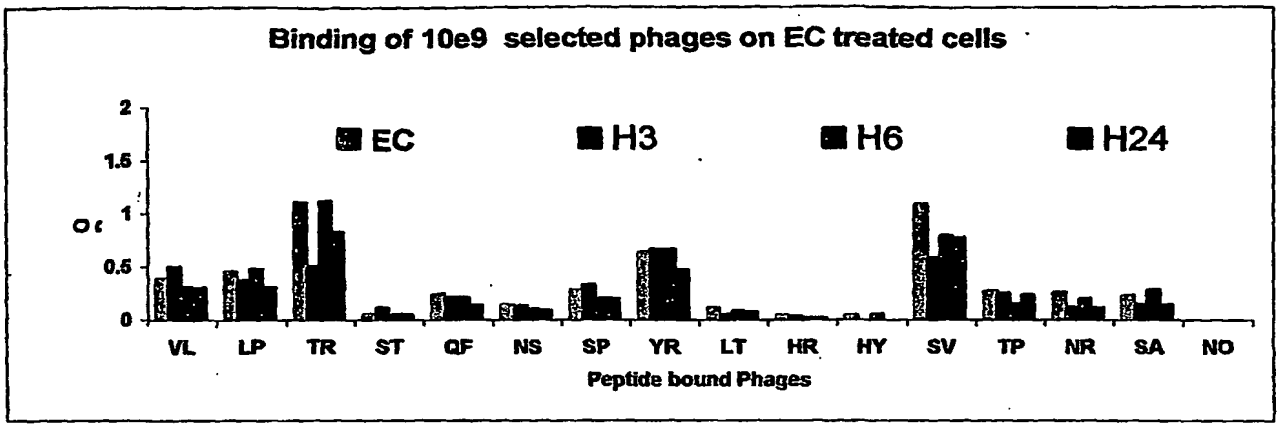
Claims:

1. A peptide as described and exemplified herein.
2. A pharmaceutical composition comprising the peptide of claim 1.
3. The peptide of claim 1, having angiogenesis inducing activity.
4. A method of inducing angiogenesis in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the peptide of claim 1 or the pharmaceutical composition of claim 2.

# Figures

**Fig.1**



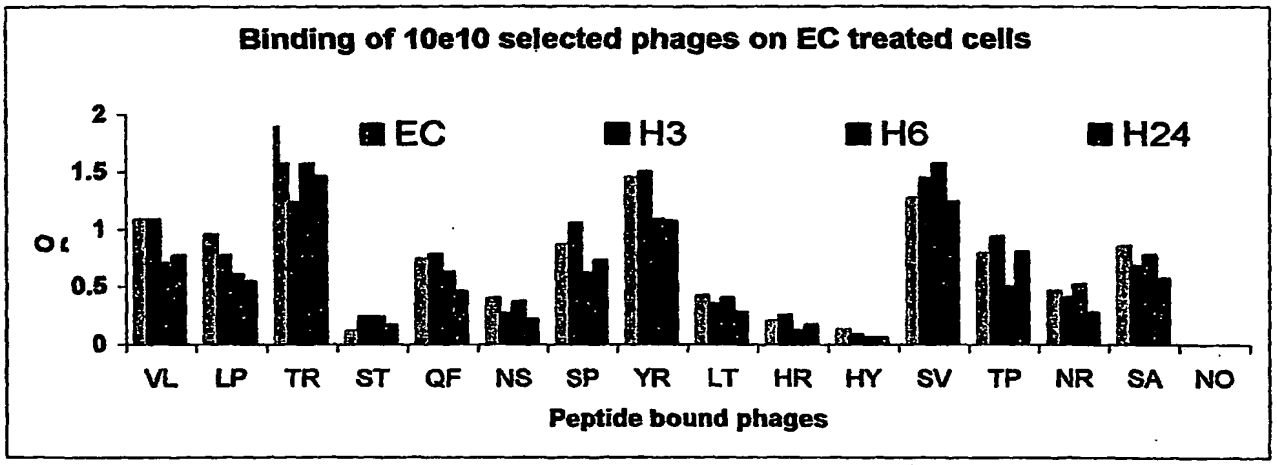



Fig. 2

No. of id ntical s quences obtained from positive select d phag s

| Cells | EC | Hx3h | Hx24h |
|---|---|---|---|
| VL | 22 | - | 10 |
| LP | 2 | 29 | 9 |
| TR | 2 | - | 2 |
| ST | 2 | - | - |
| QF | 4 | - | - |
| NS | 2 | - | - |
| SP | - | 3 | 2 |
| YR | - | 2 | - |
| LT | - | - | 4 |
| HR | - | - | 3 |
| HY | - | - | 2 |
| TP | - | - | - |
| NR | - | - | - |
| SA | - | - | - |

Fig.3

| Selected Peptides | In short |
|---|---|
| V P W M E P A Y Q R F L<br>gttccgtggatggagccggcttatcagaggtttctg | VL |
| L P A D T F E H R F W T<br>ctgcttgcggatacgacgcatcataggccgtggact | LT |
| Q P W L E Q A Y Y S T F<br>cagccttggttggagcaggcttattatagtacgttt | QF |
| S A H G T S H T G V P W P<br>tctgcgcatgggacgtctactggtgttccgtggccg | SP |
| Y P H I D S L G H W R R<br>tatccgcatattgattcgcttggtcattggcggcgg | YR |
| T L P W L E E S Y W R P<br>actttgccgtggctggaggagtcttattggcgtcct | TR |

Fig.4

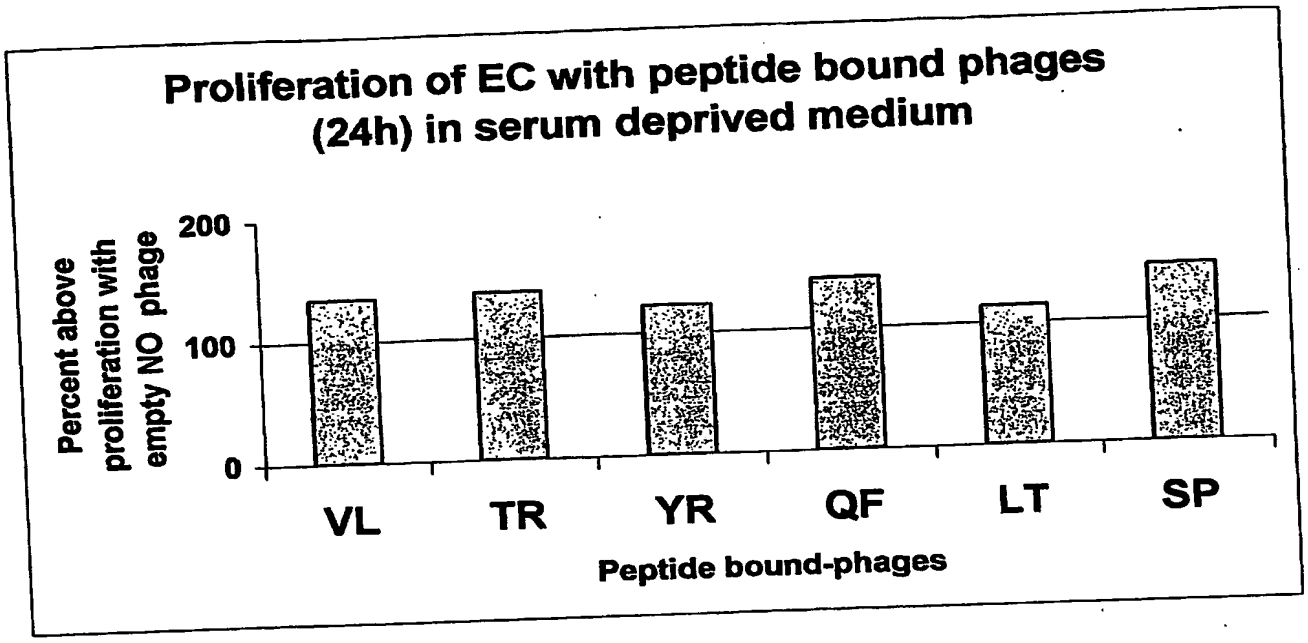
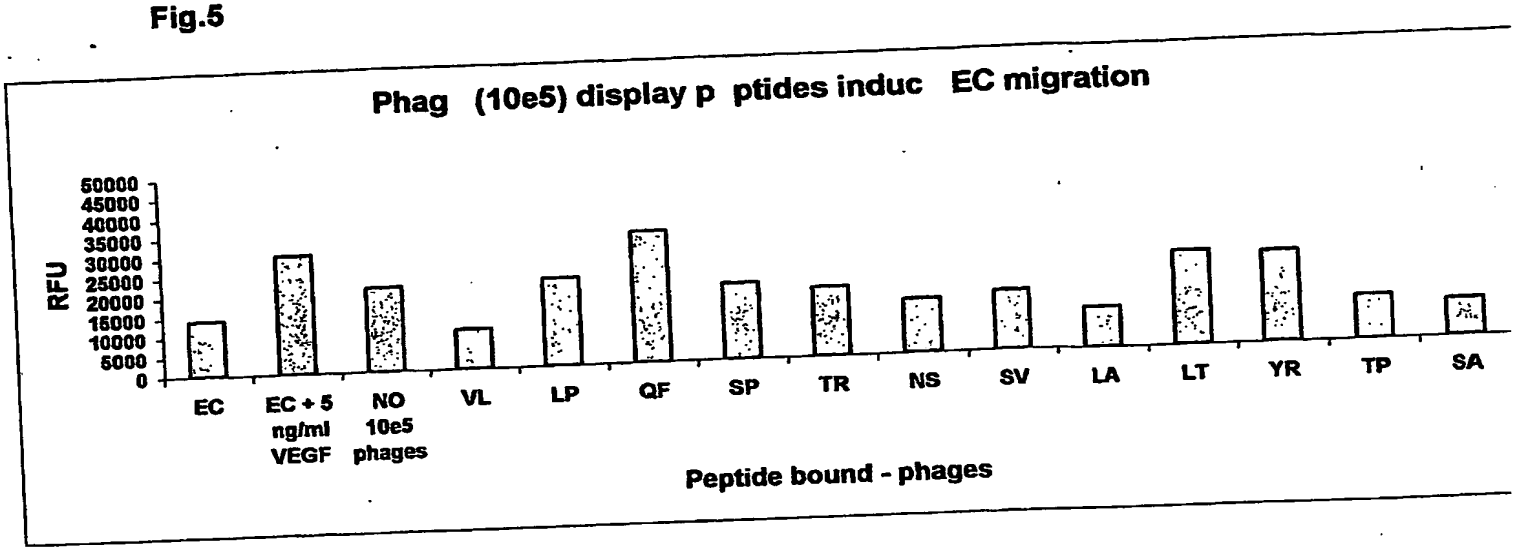

Proliferation of EC with peptide bound phages (24h) in serum deprived medium
Percent above proliferation with empty NO phage
200
100
0
VL
TR
YR
QF
LT
SP
Peptide bound-phages

Fig.5


Phag (10e5) display p ptides induc EC migration
RFU
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
EC
EC + 5 ng/ml VEGF
NO 10e5 phages
VL
LP
QF
SP
TR
NS
SV
LA
LT
YR
TP
SA
Peptide bound - phages

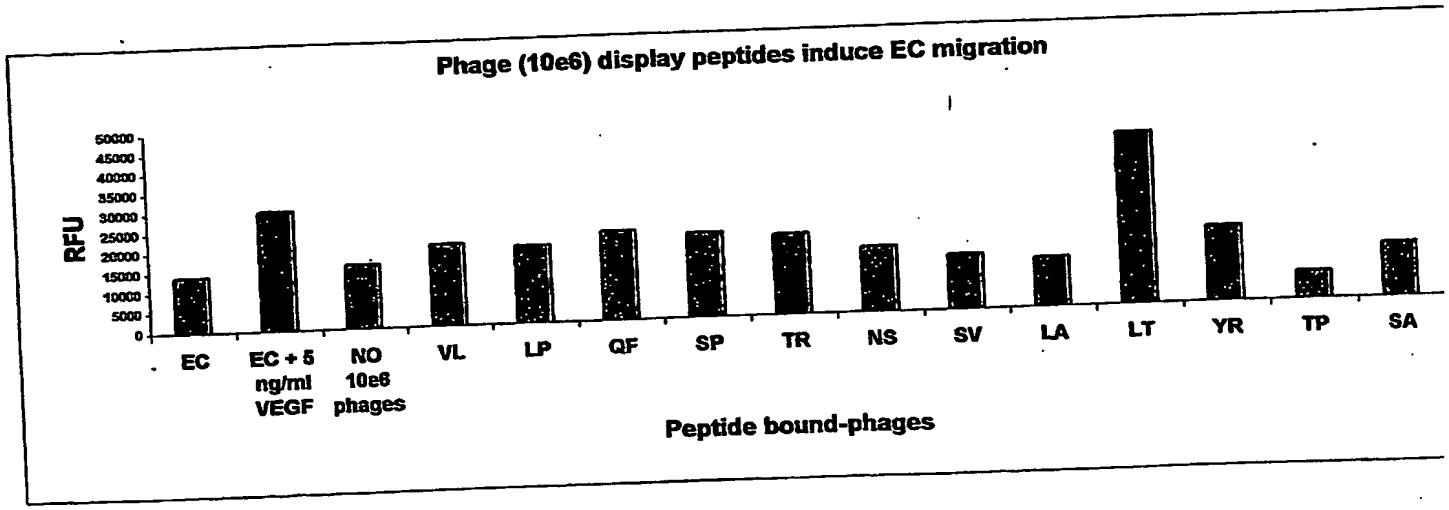
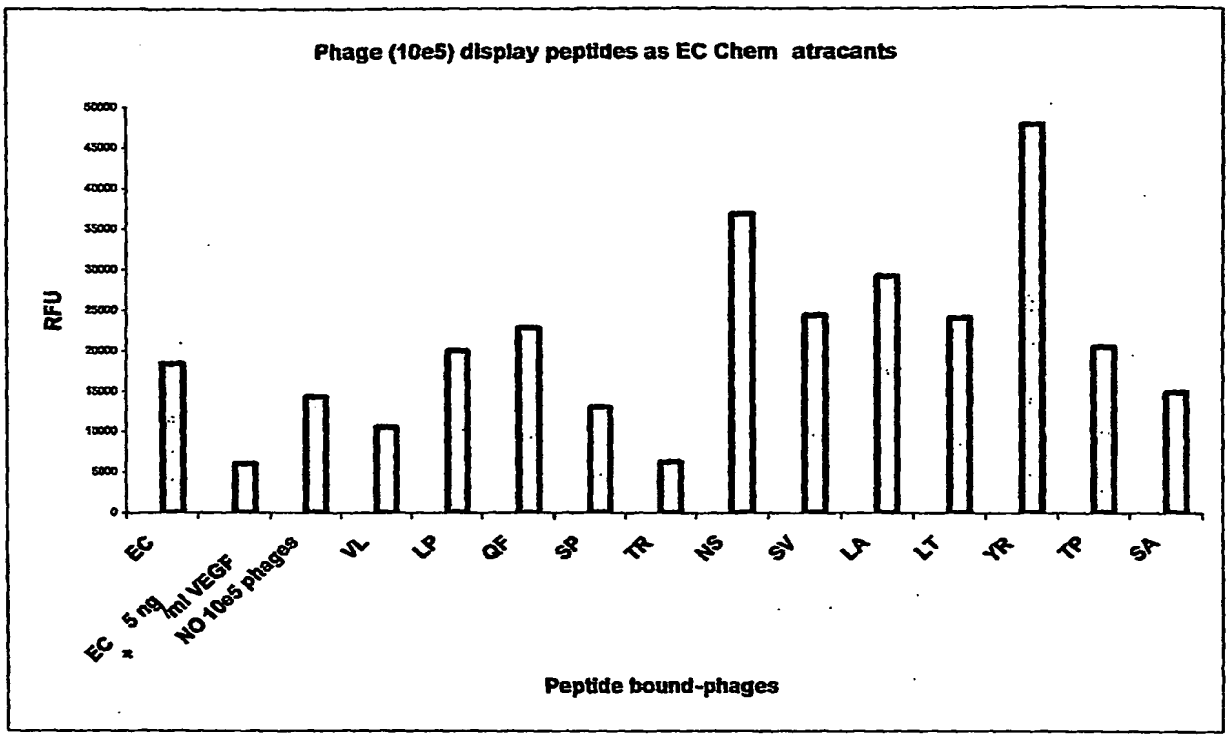

Phage (10e6) display peptides induce EC migration
RFU
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
EC
EC + 5 ng/ml VEGF
NO 10e6 phages
VL
LP
QF
SP
TR
NS
SV
LA
LT
YR
TP
SA
Peptide bound-phages

Fig.6


Phage (10e5) display peptides as EC Chem atracants
RFU
EC
EC + 5 ng/ml VEGF
NO 10e5 phages
VL
LP
QF
SP
TR
NS
SV
LA
LT
YR
TP
SA
Peptide bound-phages

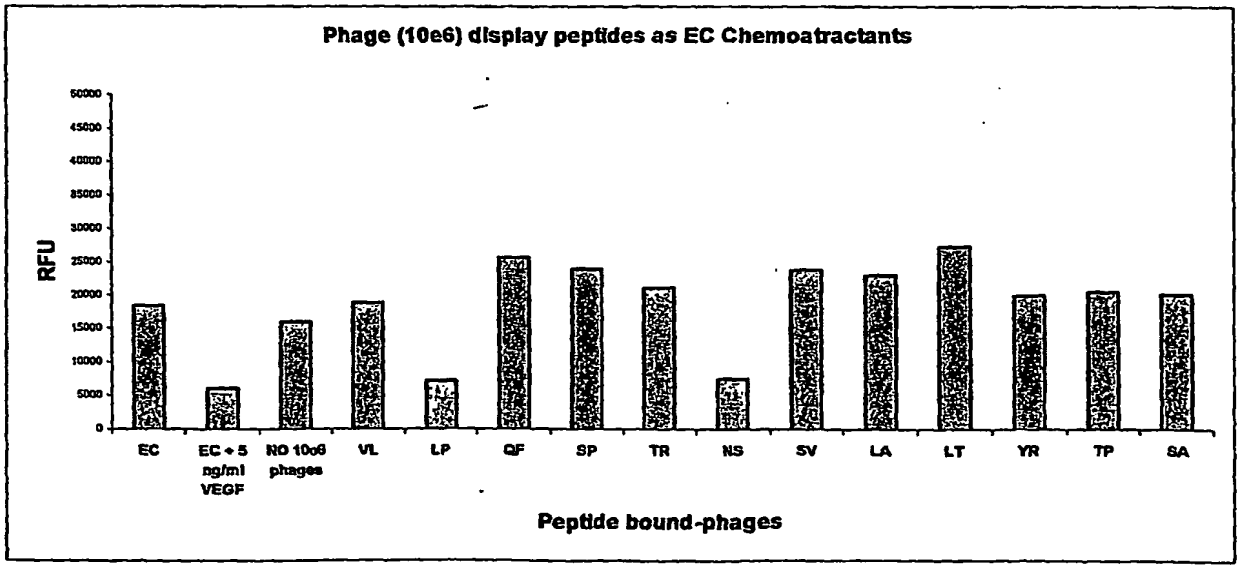

Phage (10e6) display peptides as EC Chemoatractants
RFU
EC
EC + 5 ng/ml VEGF
NO 10e6 phages
VL
LP
QF
SP
TR
NS
SV
LA
LT
YR
TP
SA
Peptide bound-phages

Fig.7

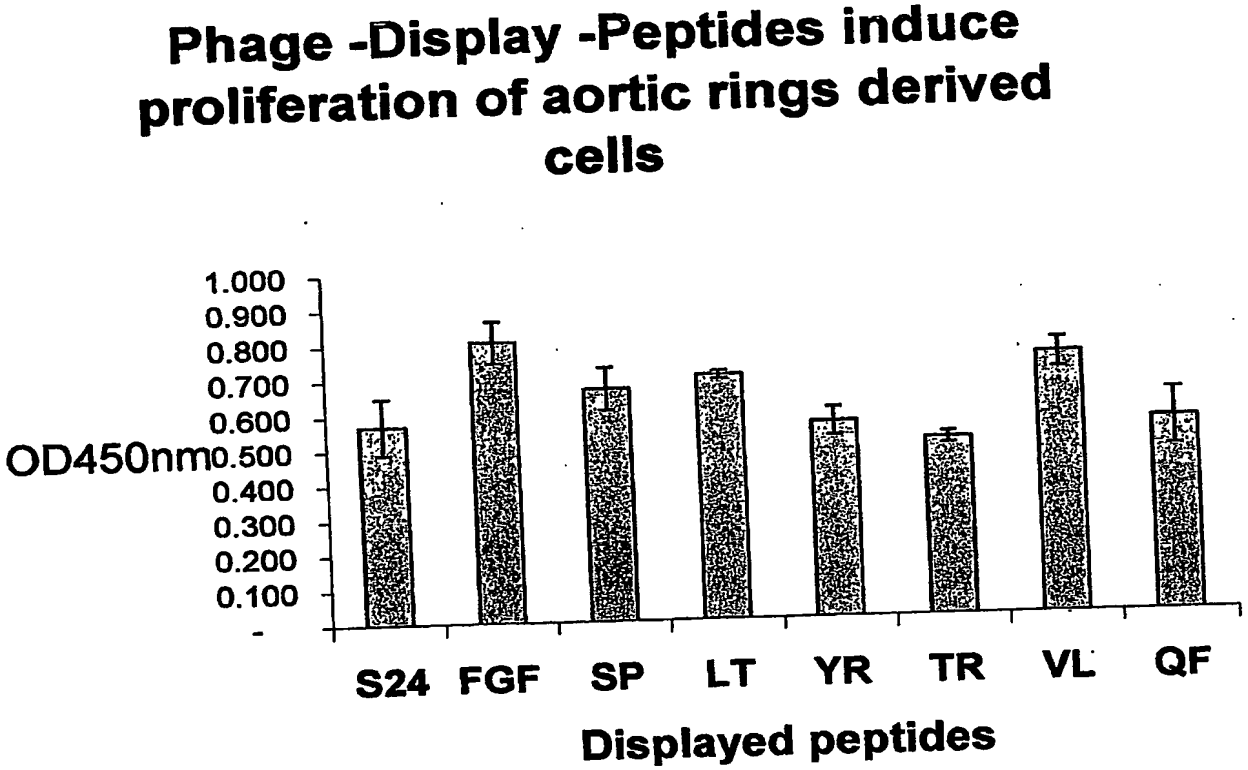

Phage -Display -Peptides induce proliferation of aortic rings derived cells
OD450nm
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
-
S24
FGF
SP
LT
YR
TR
VL
QF
Displayed peptides

Fig.8

**FACS analysis of P ptid s binding to EC**

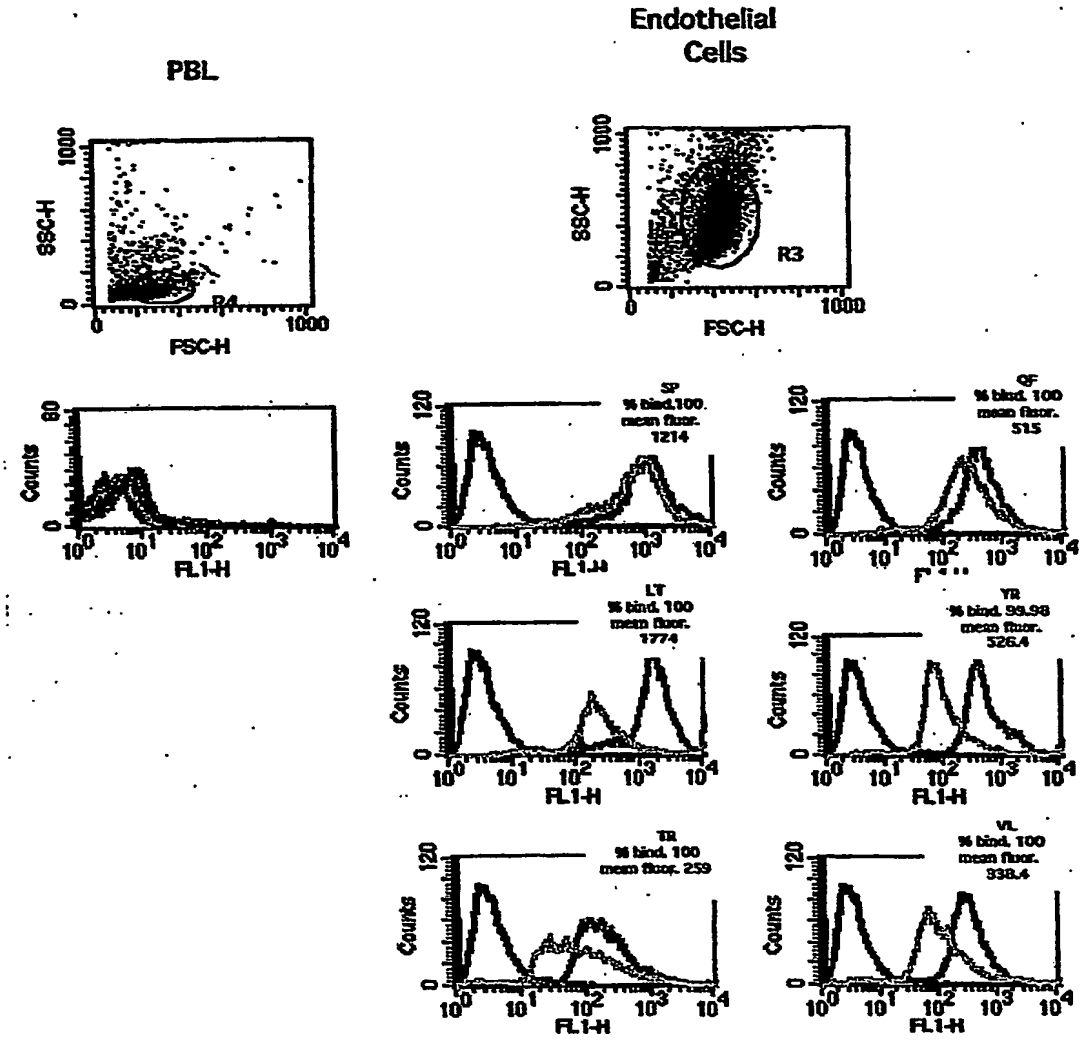


Fig.9

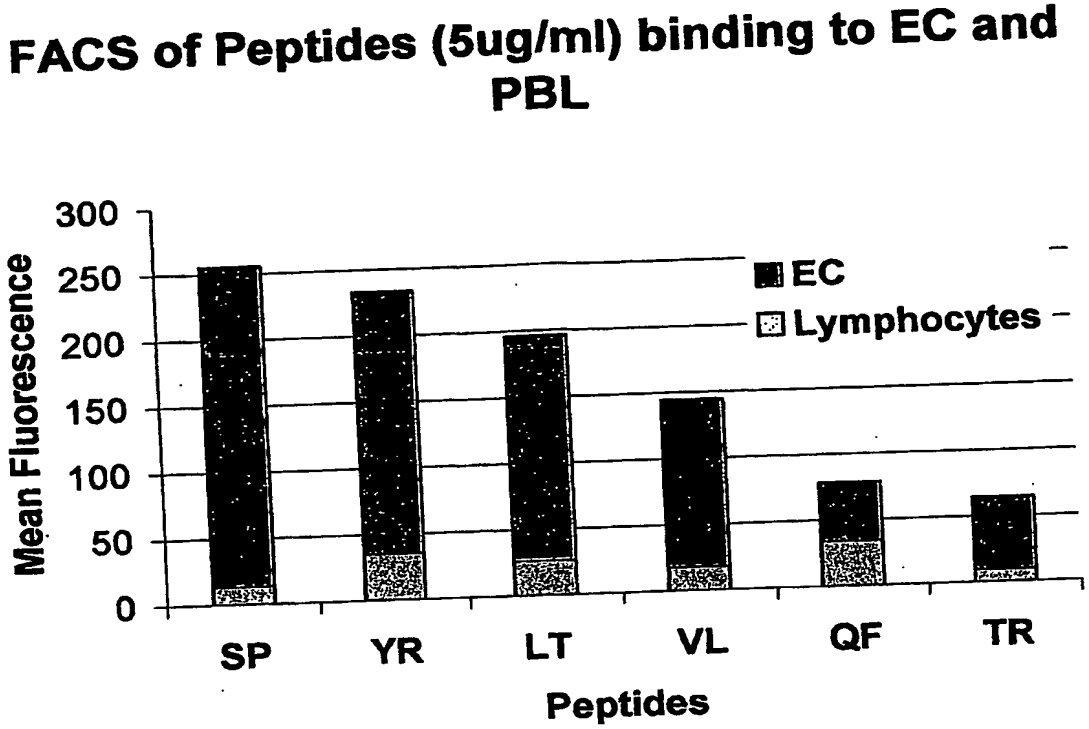

FACS of Peptides (5ug/ml) binding to EC and PBL
Mean Fluorescence
300
250
200
150
100
50
0
EC
Lymphocytes
SP
YR
LT
VL
QF
TR
Peptides

Fig. 10

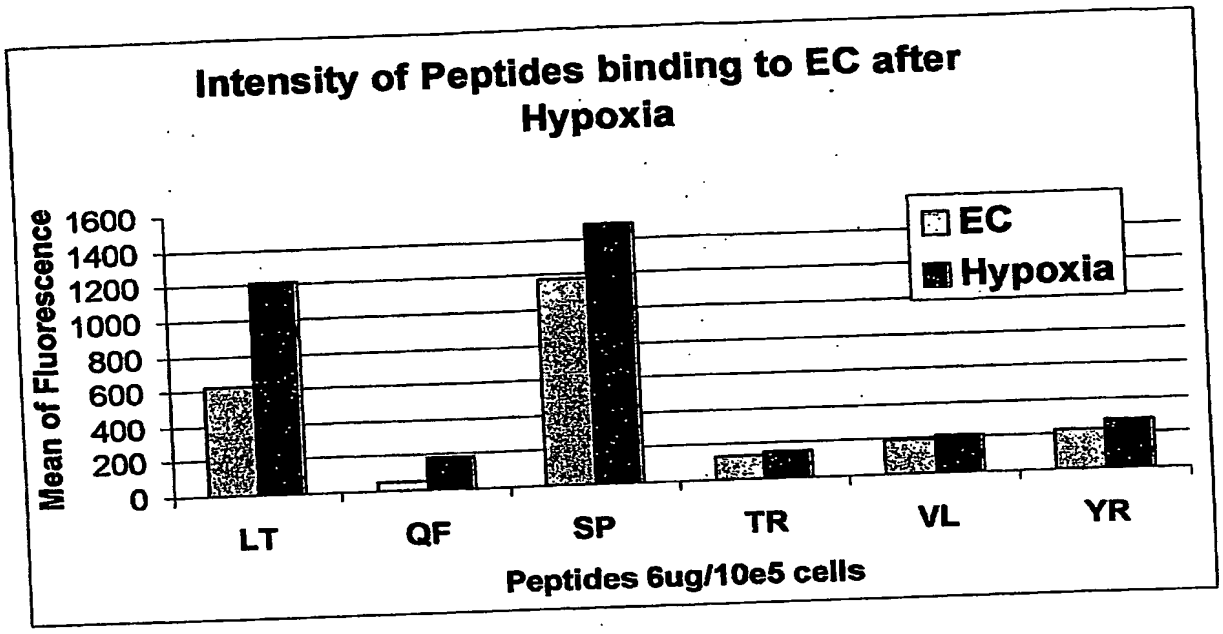

Intensity of Peptides binding to EC after Hypoxia
Mean of Fluorescence
1600
1400
1200
1000
800
600
400
200
0
EC
Hypoxia
LT
QF
SP
TR
VL
YR
Peptides 6ug/10e5 cells

**Fig.11**

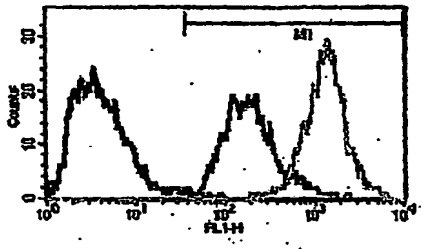
**FACS analysis of FITC lab l d SP and LT binding to EC and EC after hypoxia.**

SP-FITC binding to EC


without hypoxia
mean fluorescence: 806

with hypoxia
mean fluorescence: 1072

LT-FITC binding to EC




without hypoxia
mean fluorescence: 311

with hypoxia
mean fluorescence: 1703

Fig.12a

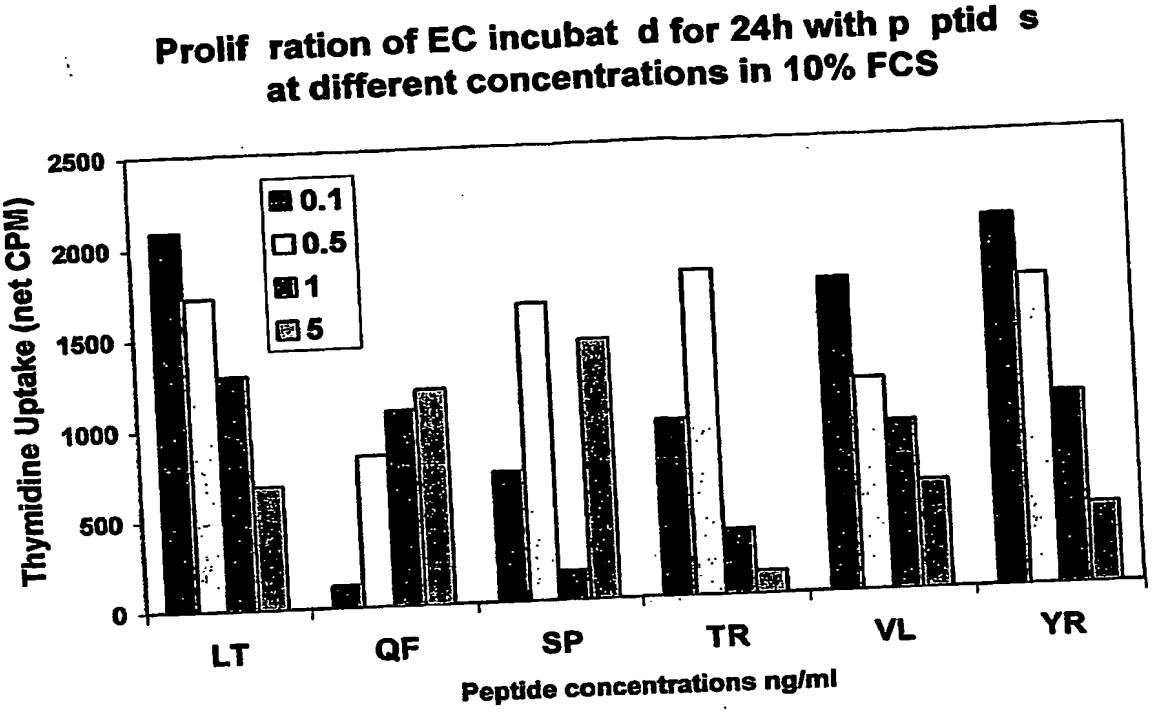

Prolif ration of EC incubat d for 24h with p ptid s
at different concentrations in 10% FCS
Thymidine Uptake (net CPM)
2500
2000
1500
1000
500
0
0.1
0.5
1
5
LT
QF
SP
TR
VL
YR
Peptide concentrations ng/ml

Fig. 12b

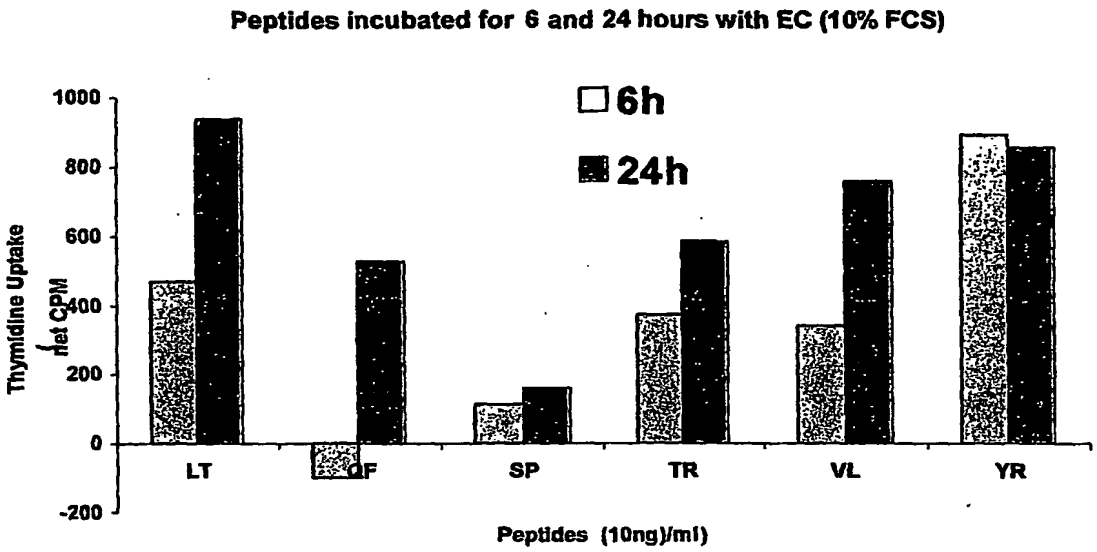

Peptides incubated for 6 and 24 hours with EC (10% FCS)
6h
24h
Thymidine Uptake
net CPM
1000
800
600
400
200
0
-200
LT
DF
SP
TR
VL
YR
Peptides (10ng)/ml)

Fig.12c

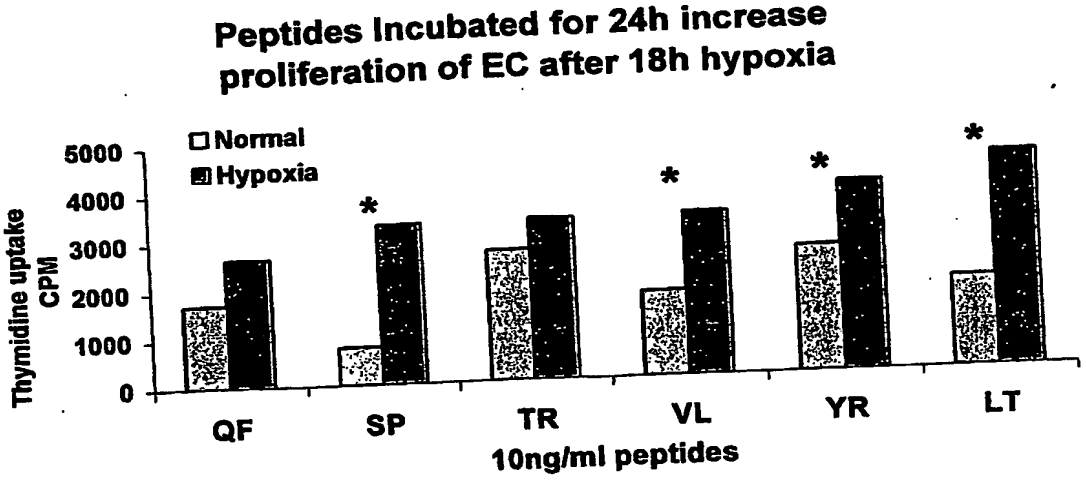

Peptides Incubated for 24h increase proliferation of EC after 18h hypoxia
Normal
Hypoxia
Thymidine uptake CPM
5000
4000
3000
2000
1000
0
*
*
*
*
QF
SP
TR
VL
YR
LT
10ng/ml peptides

Fig.13a

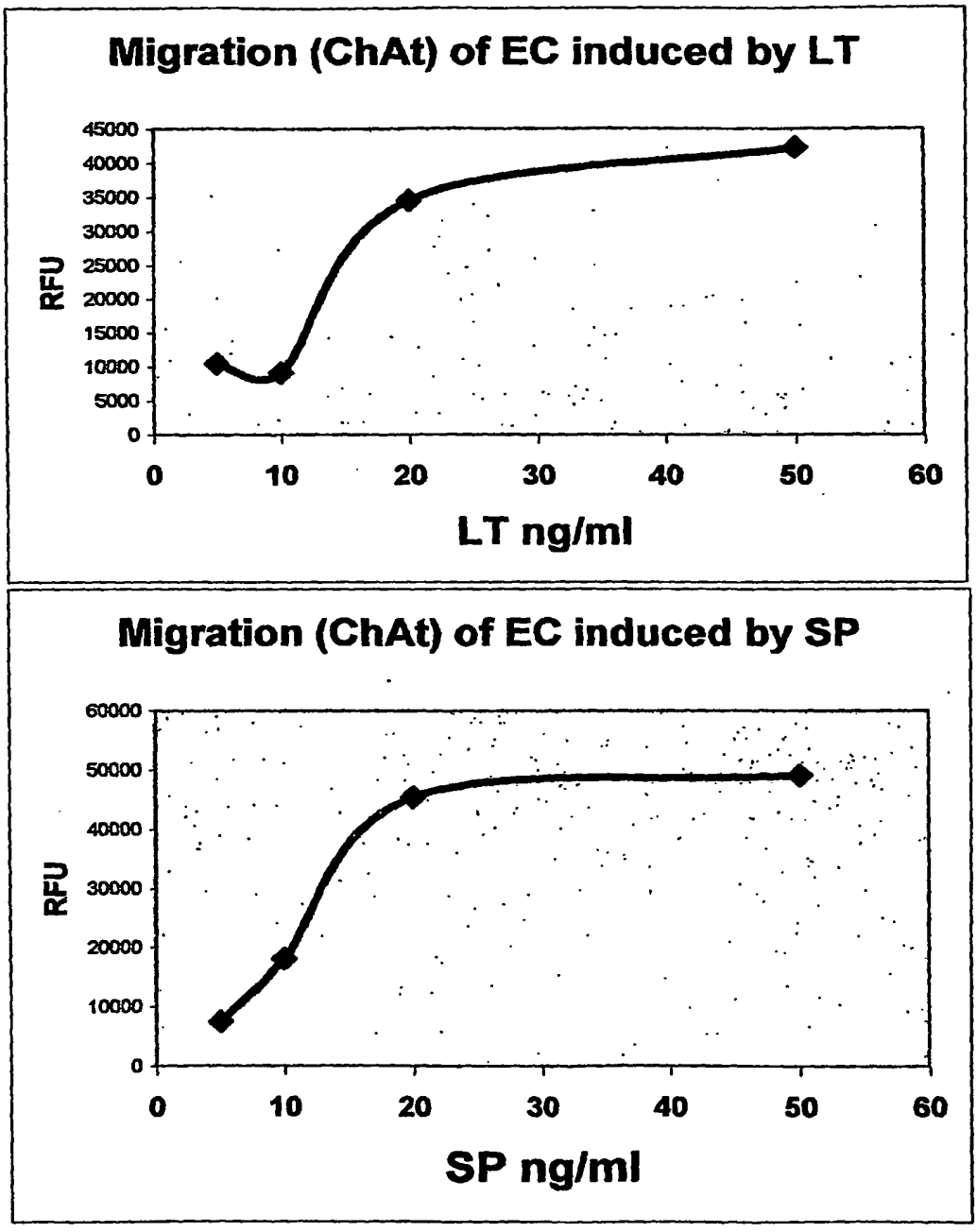

Migration (ChAt) of EC induced by LT
RFU
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
60
LT ng/ml
Migration (ChAt) of EC induced by SP
RFU
60000
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
60
SP ng/ml

Fig.13b

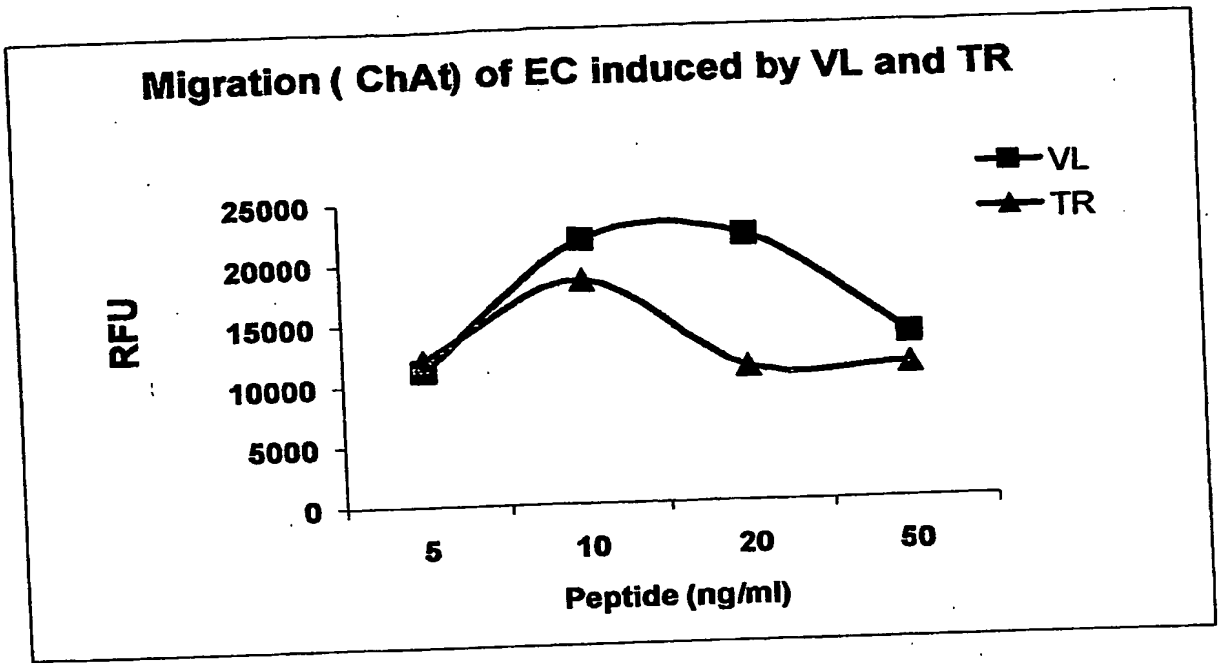

Migration ( ChAt) of EC induced by VL and TR
VL
TR
RFU
25000
20000
15000
10000
5000
0
5
10
20
50
Peptide (ng/ml)

Fig.14

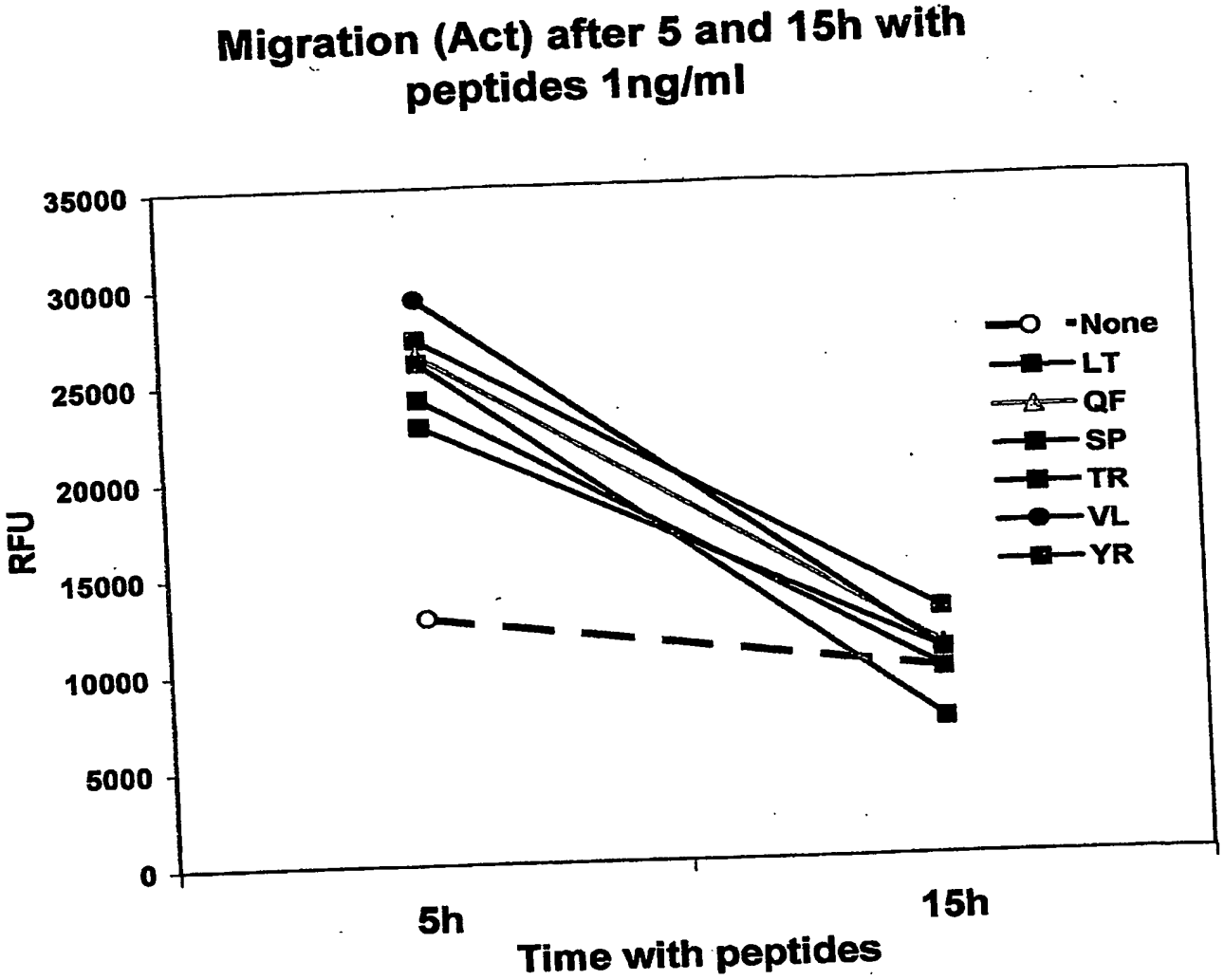

Migration (Act) after 5 and 15h with peptides 1ng/ml
35000
30000
25000
20000
15000
10000
5000
0
RFU
5h
15h
Time with peptides
None
LT
QF
SP
TR
VL
YR

Fig.15

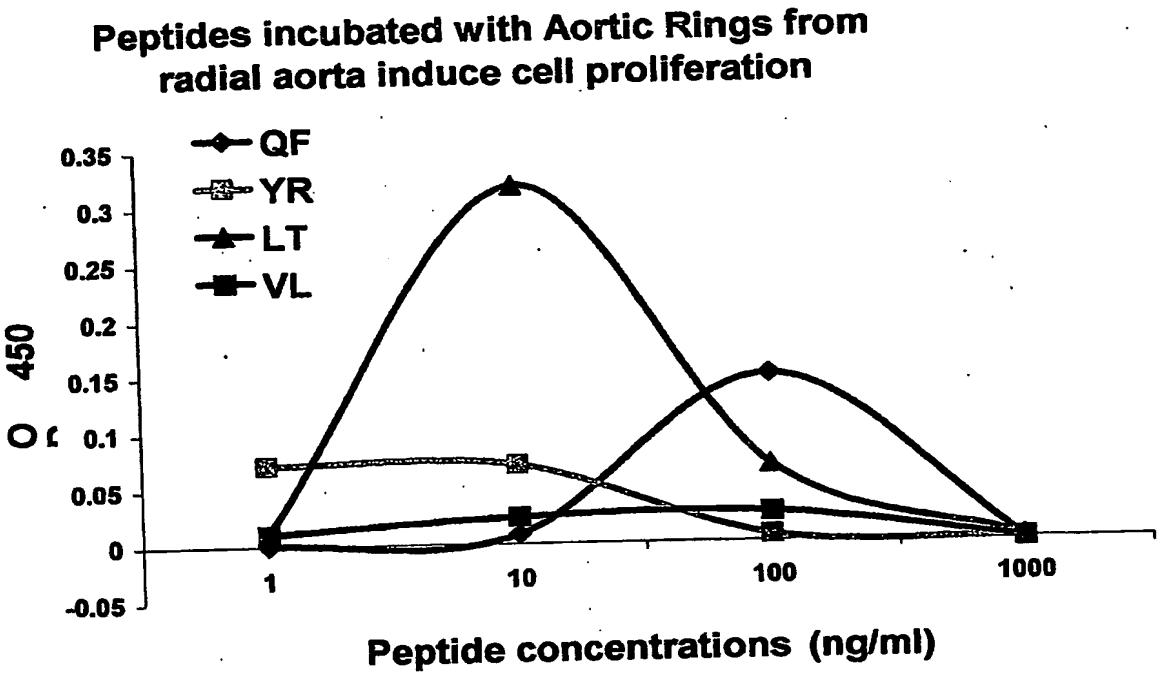

Peptides incubated with Aortic Rings from radial aorta induce cell proliferation
QF
YR
LT
VL
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
1
10
100
1000
Peptide concentrations (ng/ml)

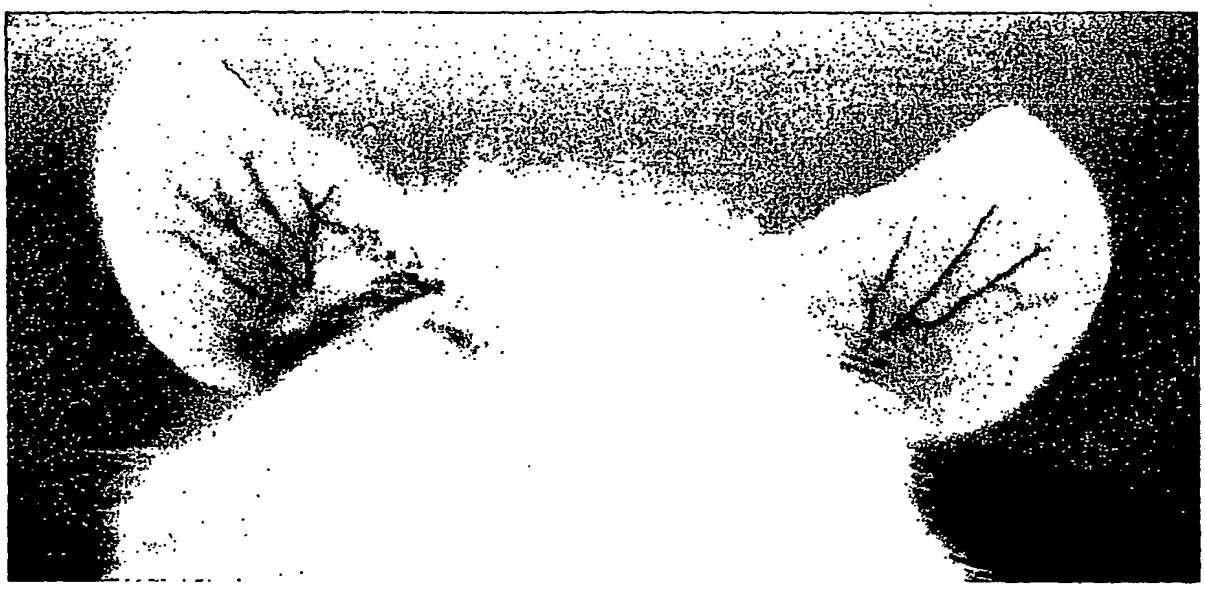
**Fig.16**

Vascularization

**The series of slides below shows vascularization induced in the ears of mice 2 days and 5 days after injection of the peptides. In each case, increases in the number of blood vessels and in branching can be observed in the injected ear compared to the control (untreated) ear.**

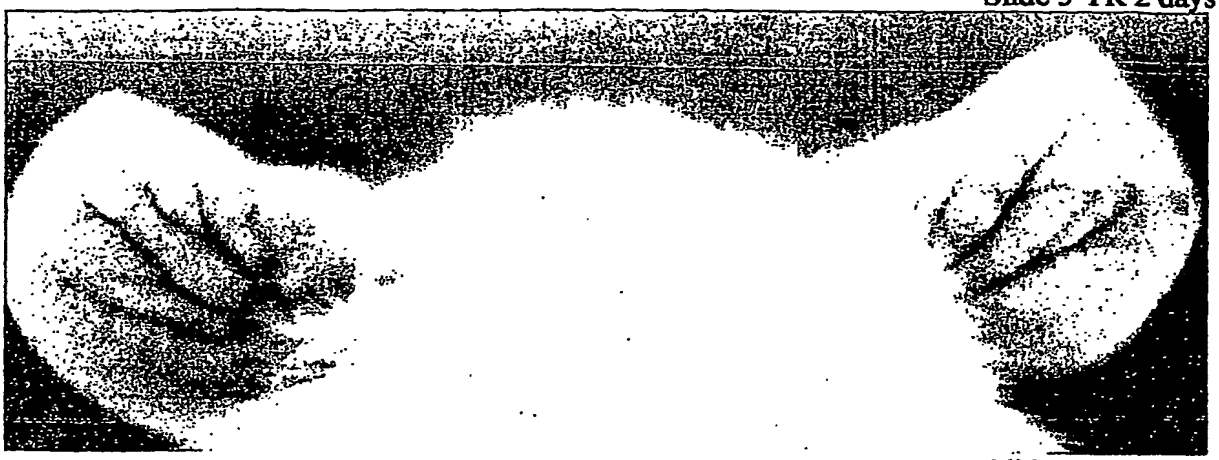
Slide 1 VEGF

Non treated ear- Control

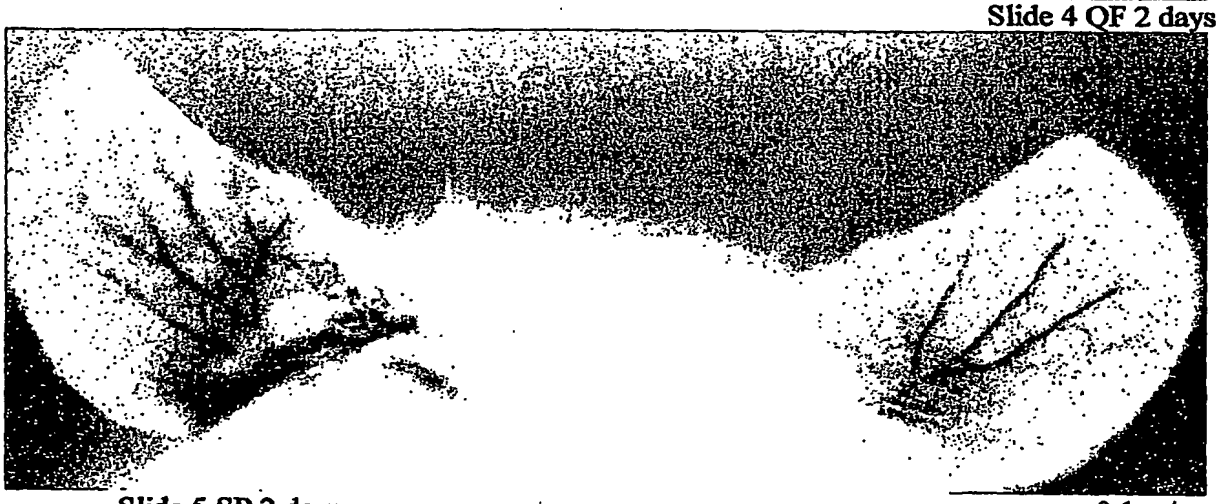


Slide 2 LT 2 days

Slide 3 YR 2 days



Slide 4 QF 2 days



Slide 5 SP 2 days

0.1ug/ear

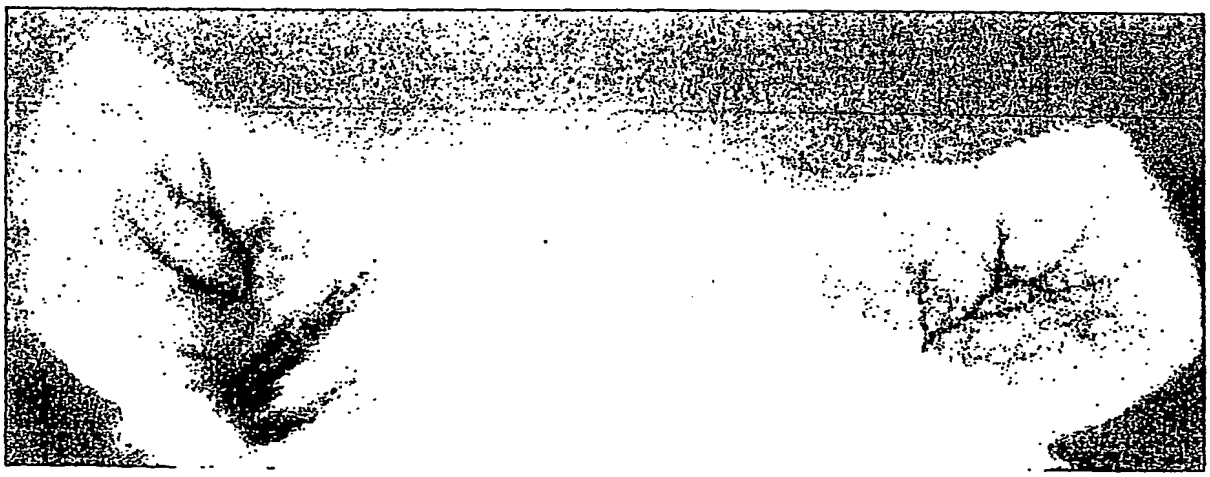


Fig.17

**Histology of mouse ear inject d with peptide: Sections tak n at th bottom and middle areas of the ears of mice injected with different peptides**

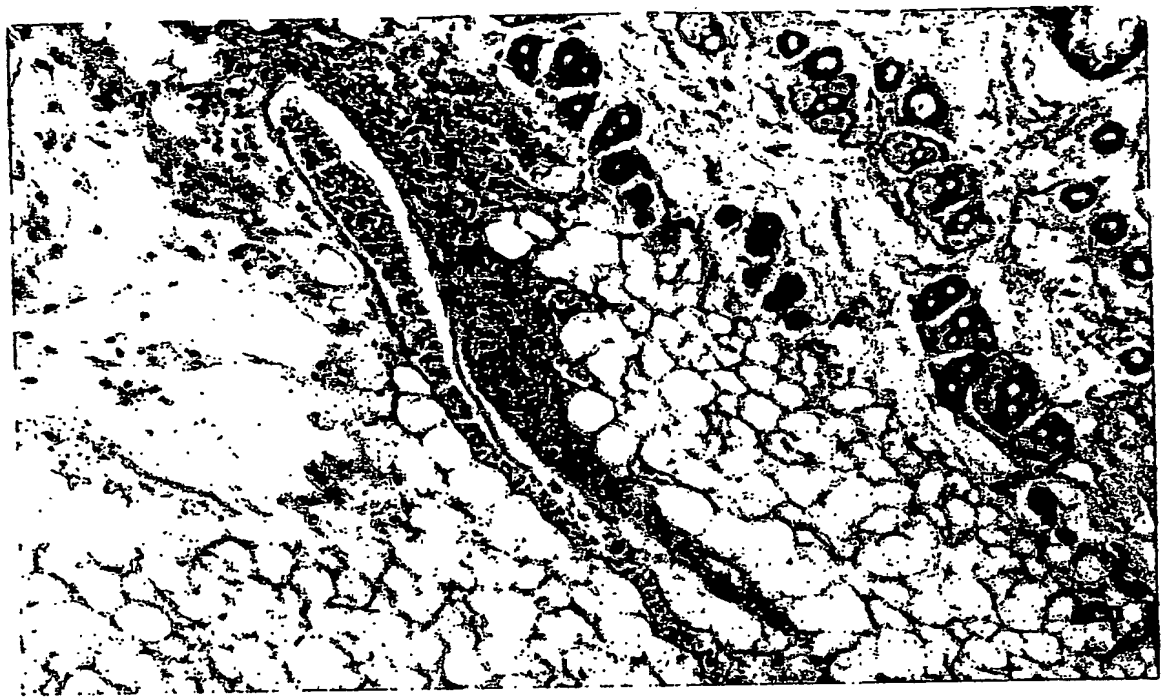


Fig.18

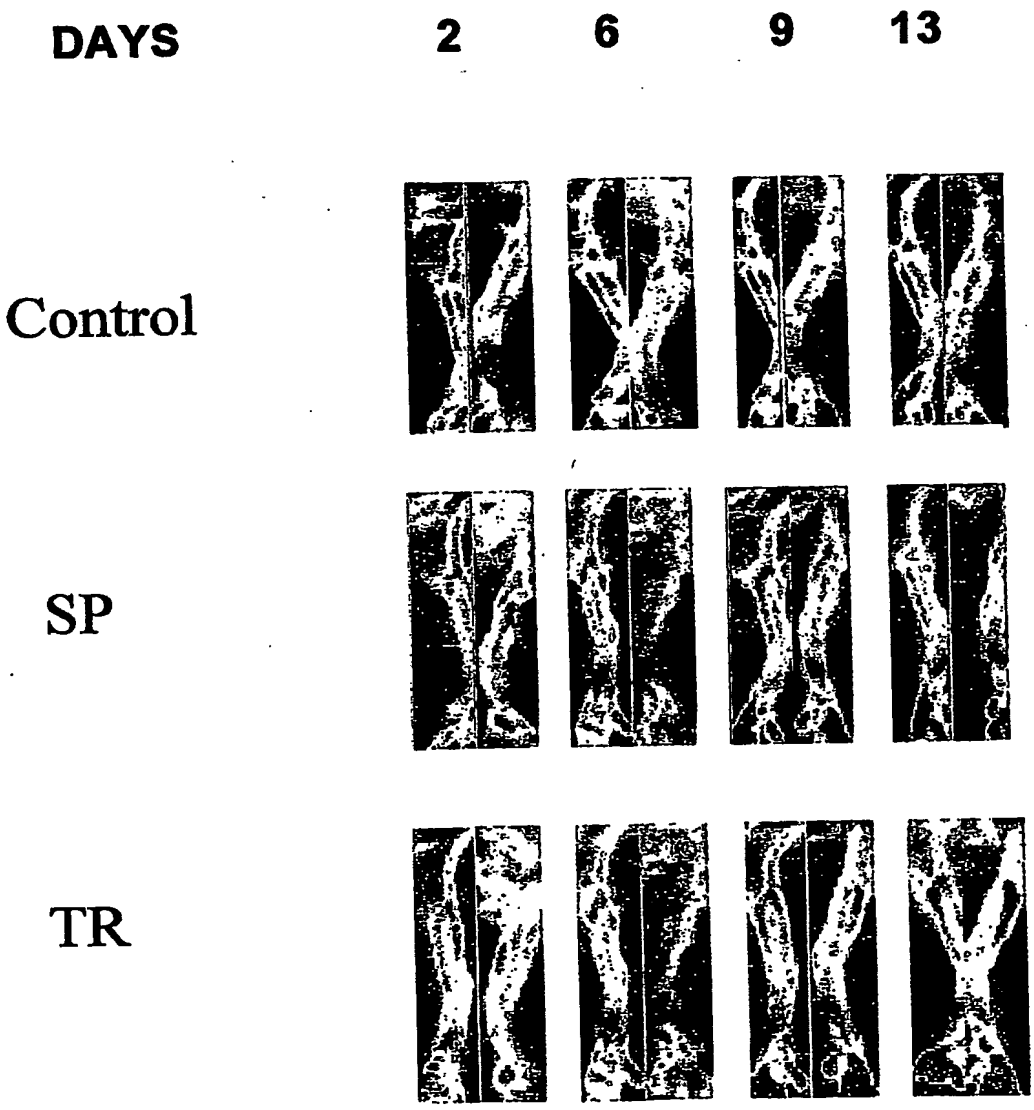
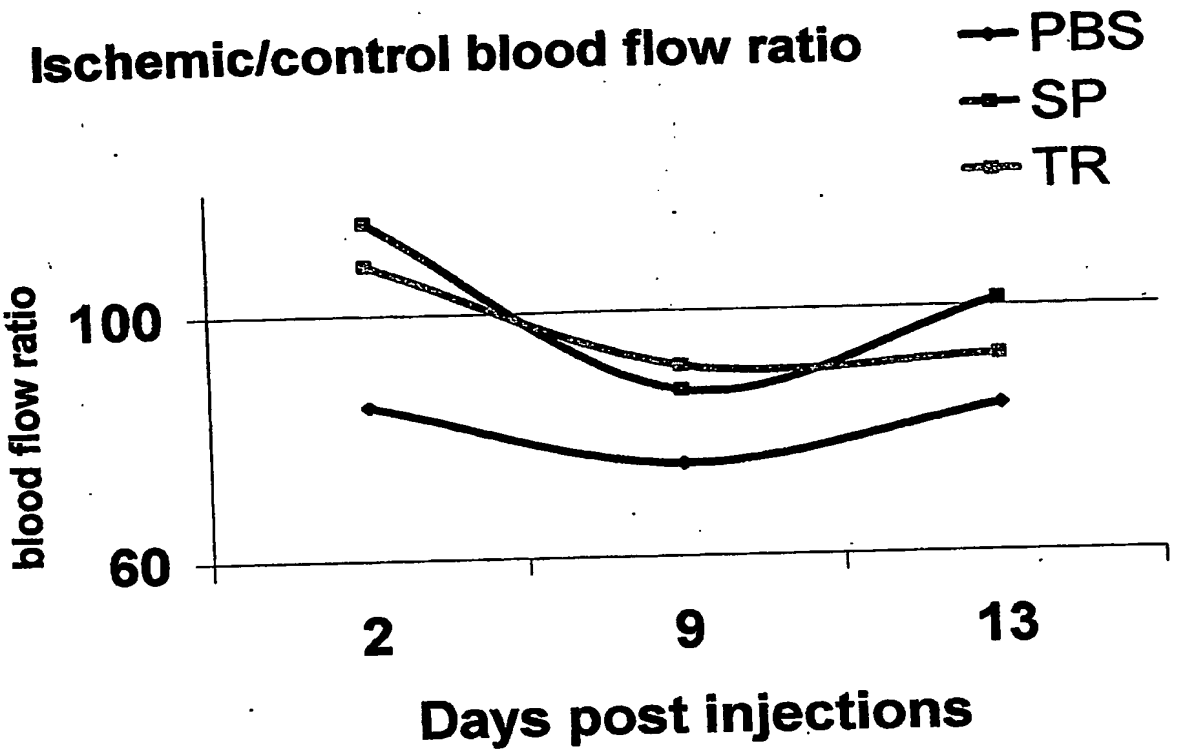


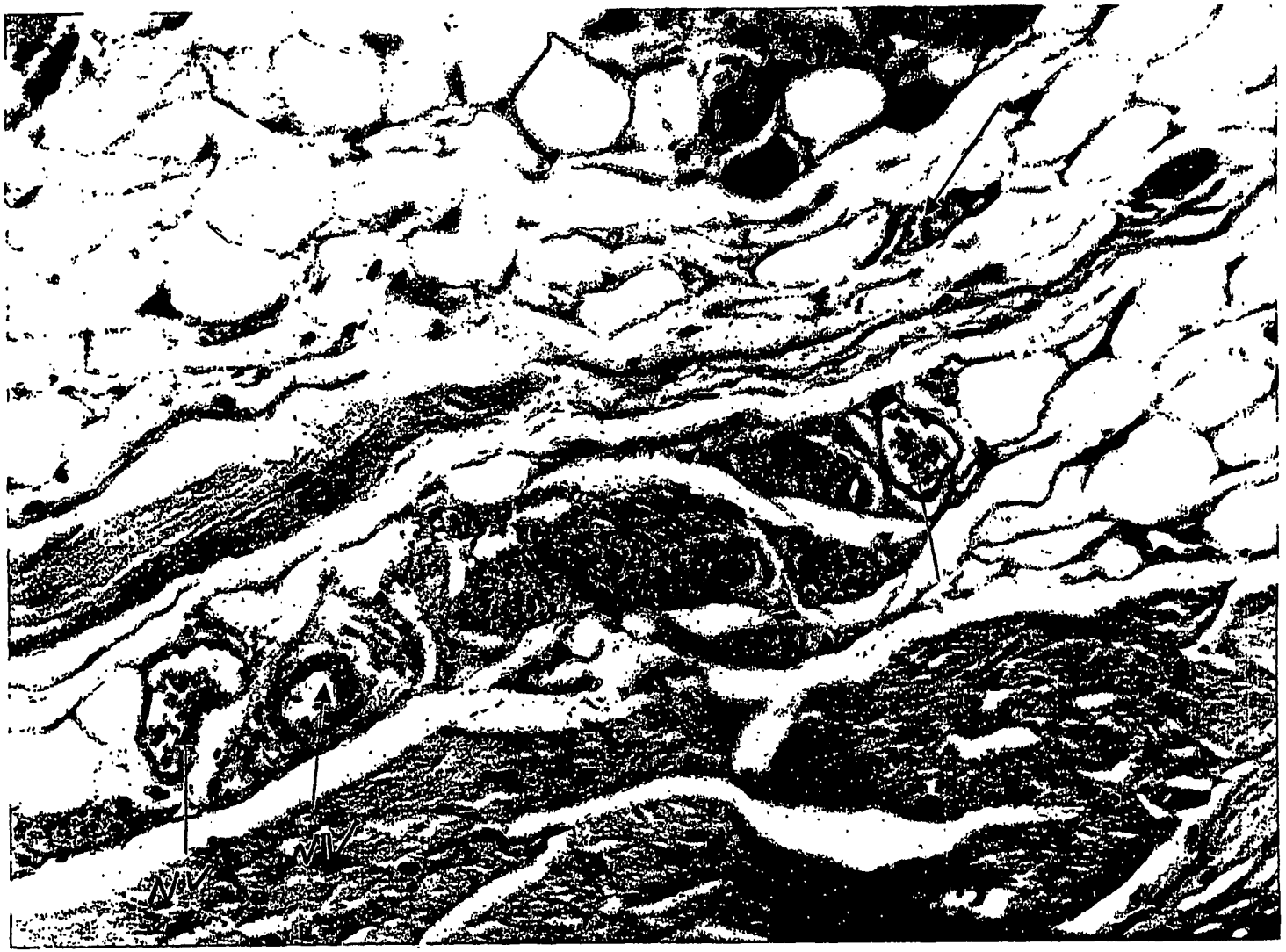
Fig.19


Ischemic/control blood flow ratio
PBS
SP
TR
blood flow ratio
100
60
2
9
13
Days post injections

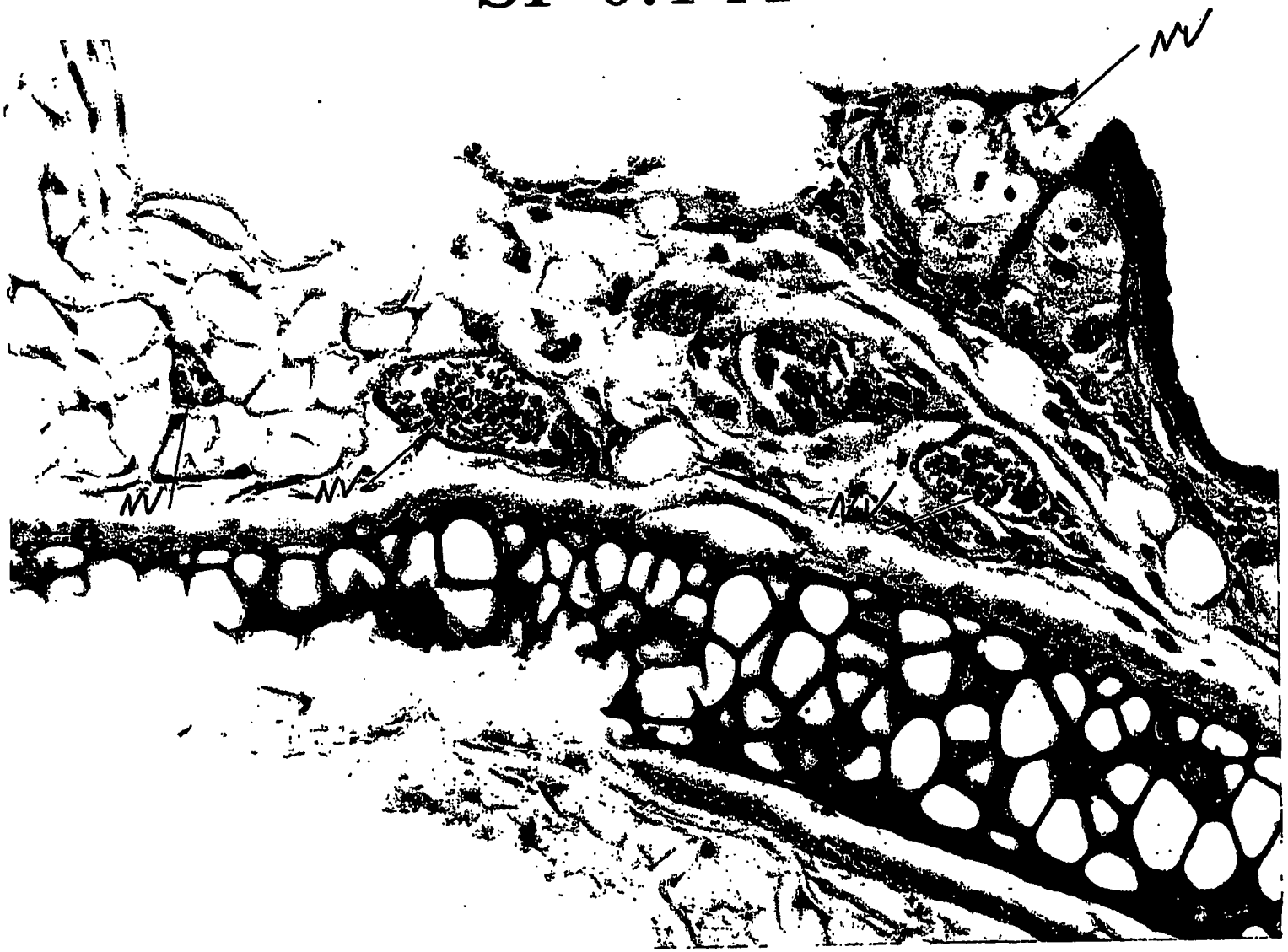
NV= Neo Vascularization

# PBS A

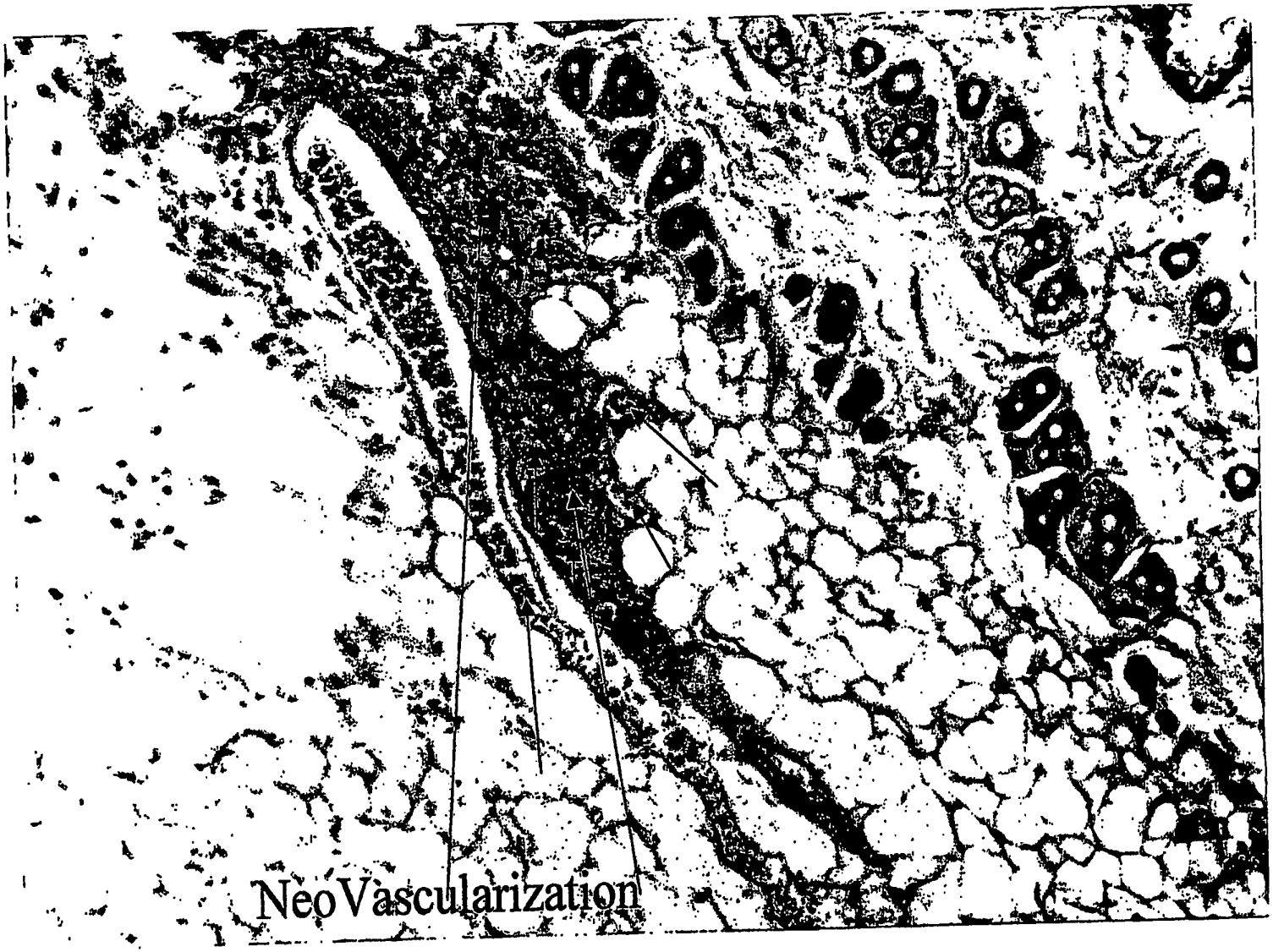
# SP 0.1 A



# SP 0.1 A

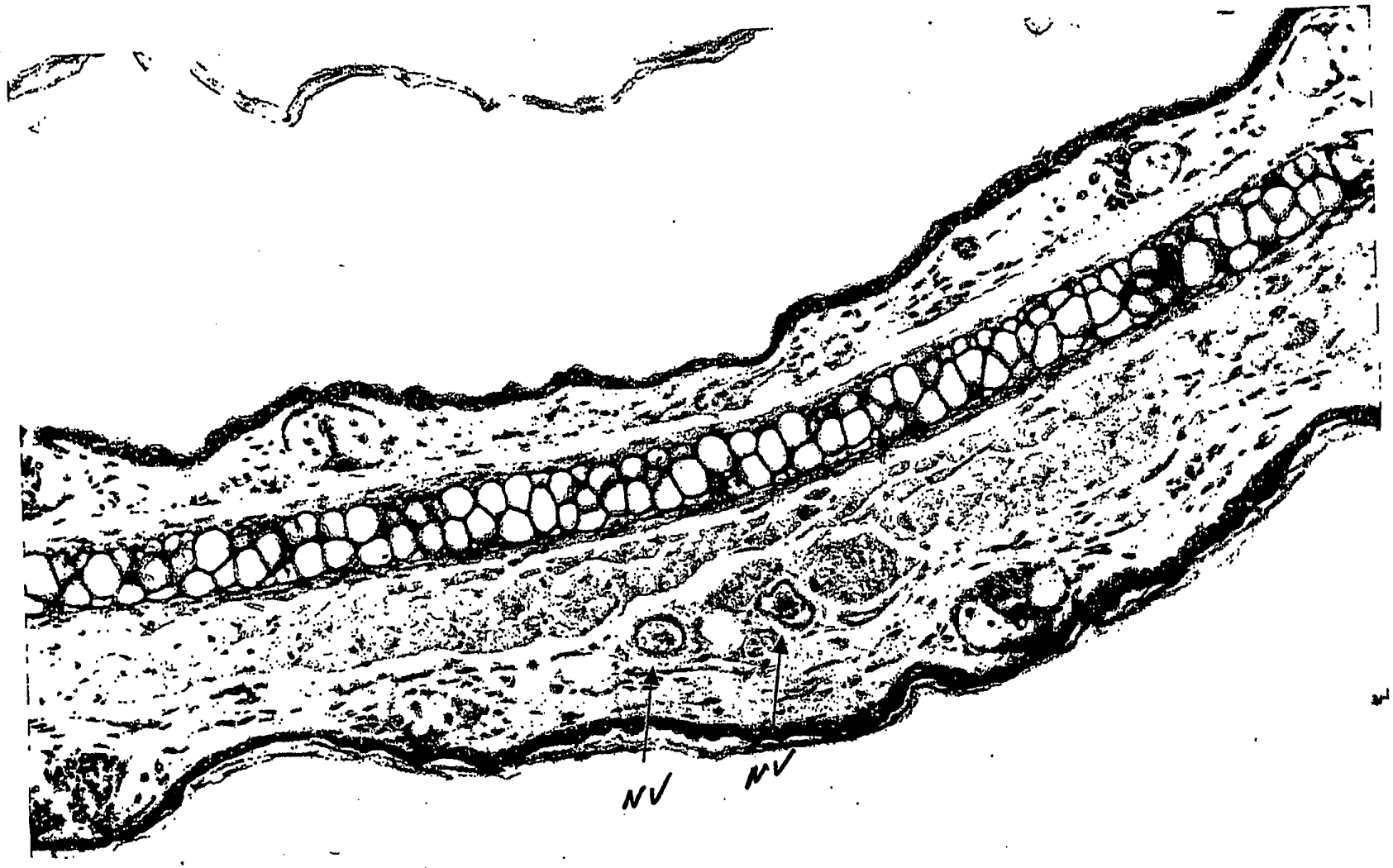


# TR 10 A

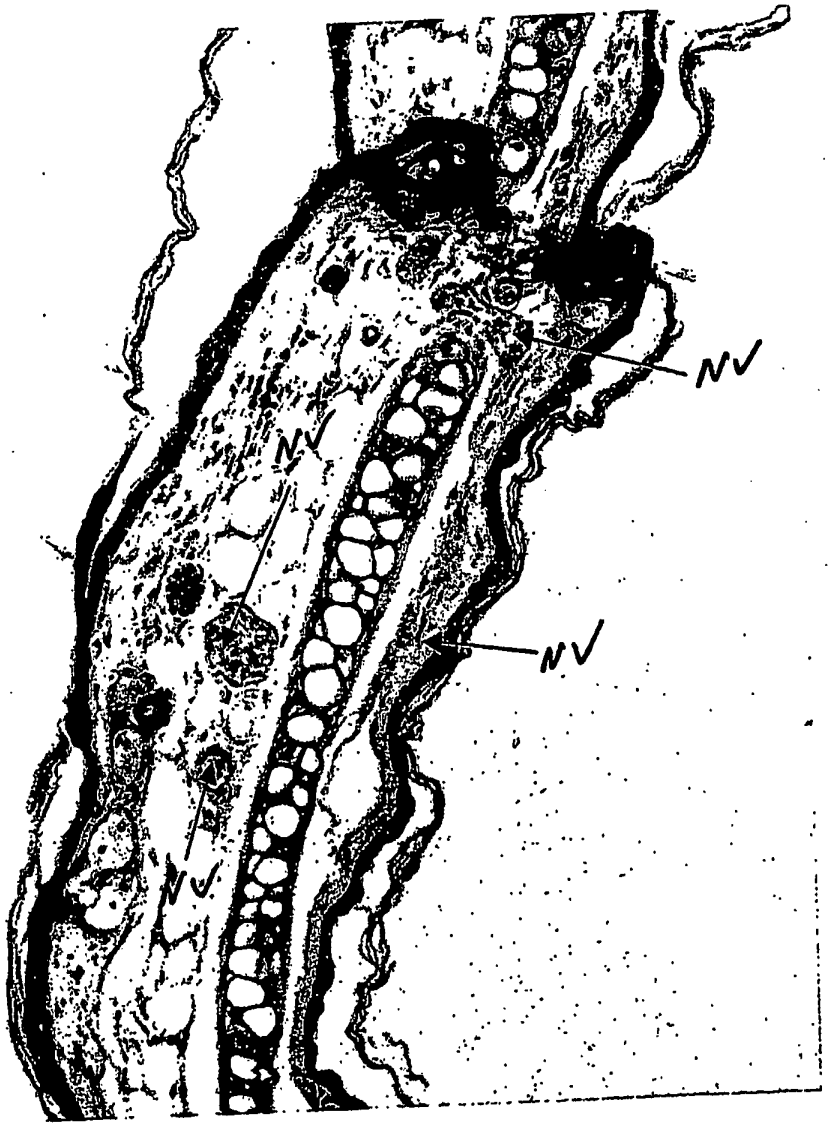


# TR 10 A

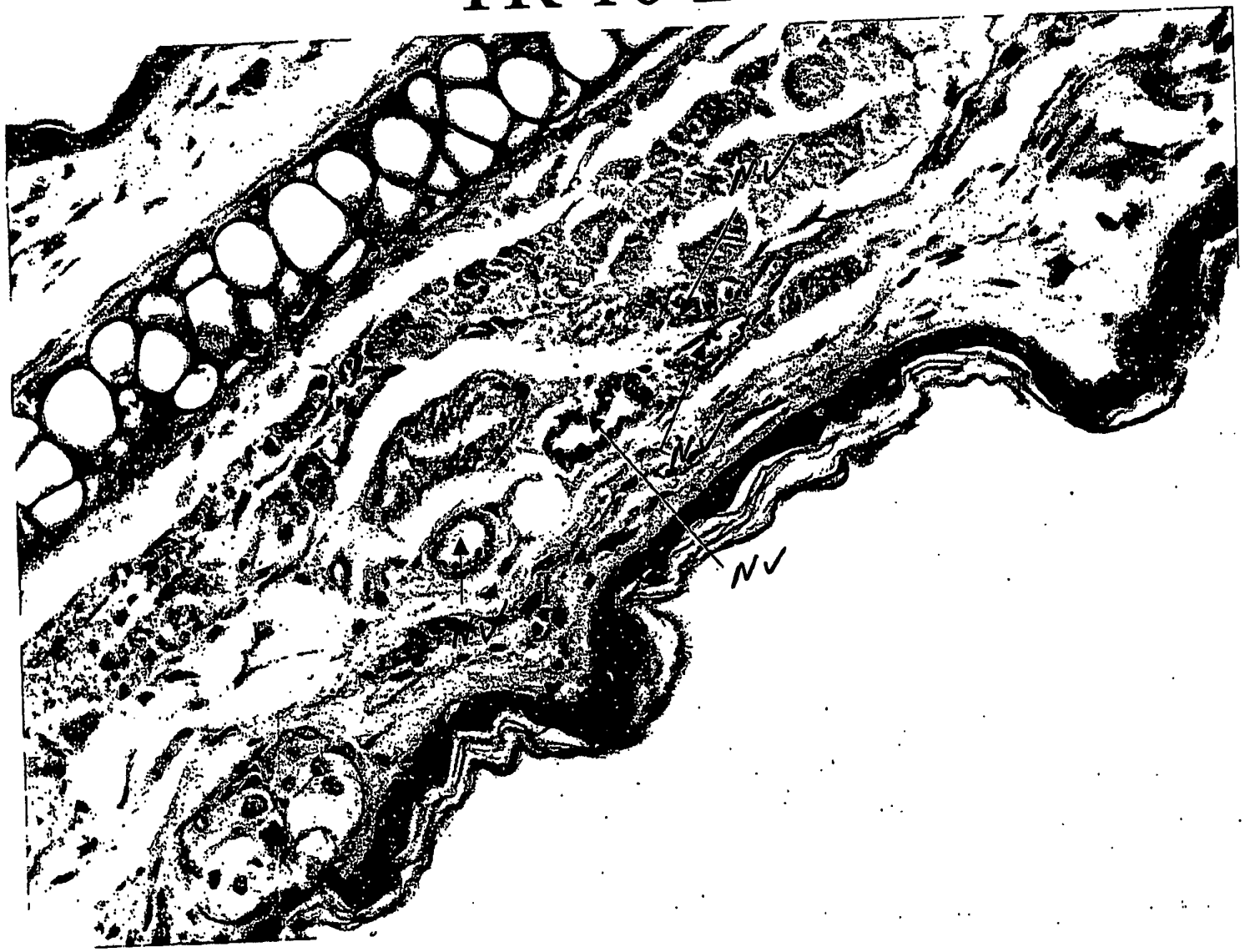
# PBS B



# TR 10 B



# TR 10 B



# SP 0.1 B

SP 0.1 B